Rom J Diabetes Nutr Metab Dis

ISSN print: 2068-8245
ISSN online: 2284-6417
ISSN-L: 2068-8245

VOLUME 27 • ISSUE 4
OCTOBER–DECEMBER 2020

Indexed by: IndexCopernicus International • getCITED • SCOPUS • Scirus

ROMANIAN JOURNAL of DIABETES, NUTRITION AND METABOLIC DISEASES

The Official Journal of the ROMANIAN SOCIETY OF DIABETES, NUTRITION AND METABOLIC DISEASES

Original Research

ORAL LICHEN PLANUS AND DIABETES: A CLINICAL STUDY
Ioanina Părlătescu, Carmen Nicolae, Roxana Oancea, Cristian Funieru

MITOCHONDRIAL DYSFUNCTION OF MESENCHYMAL STEM CELLS ISOLATED FROM BLOOD WITH TYPE 2 DIABETIC PATIENTS
Svetlana Mykolaivna Gramatiuk, Irina Yuriivna Bagmut, Michael Ivanivich Sheremet, Julia Viktorivna Ivanova, Igor Leonidovich Kolisnik, Maria Sergiivna Gramatiuk, Dragos Cretoiu, Vitaliy Vasilyevich Maksymyuk, Volodimir Volodimirovich Tarabanchuk, Oleksandr Volodimirovich Lazaruk, Petro Vasilyevich Moroz

STUDY TO EVALUATE THE CORRELATION BETWEEN COAGULATION FACTOR, GLYCEMIC CONTROL AND THE SEVERITY OF DIABETIC FOOT ULCERS AMONG SOUTH INDIAN POPULATION: A CASE CONTROL STUDY
Santhini Gopalakrishnan, Anuradha G, Sumathy S, Sandeep Unnikrishnan Dhanvarshini S

DECREASE OF PLASMA TNF-A AND CRP LEVELS IN RESPONSE TO POST-EXHAUST RESISTANCE TRAINING AND VITAMIN D SUPPLEMENTATION IN OVERWEIGHT HEALTHY WOMEN
Narges Kallantar, Hoseyn Fatolahi

SUCCESSFUL DIETARY INTERVENTION PLAN FOR HASHIMOTO'S THYROIDITIS: A CASE STUDY
Nahla Subhi Al-Bayyari

Review

INHERITED OR ACQUIRED IN HYPERTENSION AND CHRONIC KIDNEY DISEASE IN DIABETES MELLITUS PATIENTS
Stoian Marilena, Dumitrache Ana Maria, Cîrciu Fivi, Stoica Victor, Radulian Gabriela

EMERGING EVIDENCE ON THE ASSOCIATION BETWEEN COVID-19 AND TYPE 2 DIABETES
Nasreem Bibi, Bahta Wara, Hana Morrissey, Patrick Ball

Sănătatea PRESS GROUP
Expertul tău în comunicare în domeniul sănătății

PUTEREA DE A REALIZA MAI MULT*[1,2]

Jardiance®
(empagliflozin)

Alegeți JARDIANCE®
pentru pacienții necontrolați glicemic aflați în tratament cu metformin

*Adițional reducerii glicemiei, JARDIANCE® a demonstrat reducerea greutății și tensiunii arteriale; JARDIANCE® nu este indicat pentru reducerea greutății sau a tensiunii arteriale.[1]

Referințe: 1. JARDIANCE Rezumatul Caracteristicilor Produsului, octombrie 2019 2. Zinman B, Inzucchi S, Lachin JM, et al. EMPA-REG OUTCOME® Rationale, design, and baseline characteristics of a randomized, placebo-controlled cardiovascular aoutcome trial of empagliflozin. Cardiovasc Diabetol. 2014;13(102):1-8.

Acest material este destinat profesioniștilor în domeniul sănătății. PC-RO-100239

Boehringer Ingelheim RCV GmbH & Co KG Viena, Sucursala București
Str. Grigore Alexandrescu nr. 89-97, Metropolis Center, Aripa Vest, et. 6,
sector 1, cod 010627, Bucharest, Romania.
Telefon: 004021-302-2800; Fax: 004021-302-2801

Boehringer Ingelheim

Romanian Journal of Diabetes, Nutrition and Metabolic Diseases

Editor-in-Chief: Cristian GUJA, MD, PhD

Carol Davila University of Medicine and Pharmacy, Bucharest, Romania
'N.C. Paulescu' National Institute of Diabetes, Nutrition and Metabolic Diseases, Bucharest, Romania,
5-7 Ion Movila Street, Bucharest 020475, Romania. Tel. +40 21 210 6460, Fax: +40 21 210 2295
E-mail: cristian.guja@b.astral.ro

Honorary Editors: Constantin Ionescu-Tîrgovişte, Maria Moţa

Editorial Board

Rodica Pop Buşui - USA
Aurel Babeş - Oradea
Doina Catrinoiu - Constanţa
Dan Mircea Cheţa - Bucharest
Mihaela Dincă - Craiova
Carmen Dobjanschi - Romania
Carol Forsblom - Finland
Alin Gîrniţă - USA
Mariana Graur - Iaşi
Per-Henrik Groop - Finland
Nicolae Hâncu – Cluj Napoca
Dumitru Lacatîş - Switzerland
Radu Lichiardopol - Bucharest
Ioana Micle - Timişoara
Dragan Micic - Serbia
Eugen Moţa - Craiova
Gabriela Negrişanu – Timişoara
Bogdan Mihai - Iaşi
Simona Georgiana Popa - Craiova
Valdis Pirags - Latvia
Amorin Remus Popa - Oradea
Gabriela Radulian - Bucharest
Gabriela Roman – Cluj Napoca
Cristian Serafinceanu - Bucharest
Viorel Şerban – Timişoara
Alin Ştirban - Germany
Romulus Timar - Timişoara
Hermann Toplak - Austria
Ioan Andrei Vereşiu – Cluj Napoca
Adrian Vlad - Timişoara

Associate Editor
Bogdan Timar, Timisoara, Romania
E-mail: bogdan.timar@rjdnmd.org

Managing Editor
Dragos Cretoiu, Bucharest, Romania
E-mail: journal@sanatateapress.ro

Assistant Editor
Roxana Stoica, Timisoara, Romania
E-mail: roxana.stoica@rjdnmd.org

Assistant Editor
Sandra Lazar, Timisoara, Romania
E-mail: sandra.lazar@rjdnmd.org

Assistant Editor
Teodor Salmen, Bucharest, Romania
E-mail: teodor.salmen@rjdnmd.org

For submission instructions and updated information, please register at: https://www.rjdnmd.org

Romanian Journal of Diabetes, Nutrition and Metabolic Diseases is the Official Journal of the **Romanian Society of Diabetes, Nutrition and Metabolic Diseases**

ISSN-L: 2068-8245
print: 2068-8245
online: 2284-6417

Indexed by: IndexCopernicus International • getCITED • SCOPUS • Scirus

Sănătatea Press Group SRL
9C Alexandru Moruzzi street, Bucharest 031152, Romania. Tel.: +40 31 710 88 01, Fax: +40 031 710 88 02
E-mail: office@sanatateapress.ro, www.sanatateapress.ro

Although the authors, together with the publisher and editors, have taken great care to ensure that information presented in the Romanian Journal of Diabetes, Nutrition and Metabolic Diseases reflects the standard of knowledge at the time of publication, in the event of an error committed either by the authors, editors, or publisher, neither the journal, nor its publisher or any other party involved in publishing the Journal claims that the information contained is in every respect precise and complete, and as such, are not responsible for errors or omissions or for the results obtained from the use of such material. Readers are encouraged to confirm the information published by the journal with other sources. The ultimate responsibility for the use and dosage of drugs mentioned in the Journal and in interpretation of published material lies with the medical practitioner, and the editors and publishers cannot accept liability for damages arising from any errors or omissions in the Journal.

Jardiance® (empagliflozin) comprimate filmate 10 mg, 25 mg. Indicaţii terapeutice: Tratamentul adulților cu diabet zaharat tip 2 insuficient controlat, ca adjuvant la dietă și exercițiul fizic: ca monoterapie atunci când metformina este considerată inadecvată din cauza intoleranței; în plus față de alte medicamente pentru tratamentul diabetului zaharat. **Doze şi mod de administrare:** Doza inițială este de 10 mg o dată pe zi (în monoterapie și în terapie asociată suplimentară cu alte medicamente pentru tratamentul diabetului zaharat). La pacienții care tolerează empagliflozin 10 mg o dată pe zi, care prezintă RFGe ≥60 ml/min/1,73 m^2 și care necesită un control glicemic mai strict, doza poate fi crescută la 25 mg o dată pe zi. Doza zilnică maximă este de 25 mg. Când empagliflozin este utilizat în asociere cu o sulfonilureе sau cu insulină, se poate avea în vedere o doză mai mică de sulfonilureе sau de insulină, pentru a reduce riscul de hipoglicemie. *IR* Nu este necesară ajustarea dozei la pacienți cu RFGe ≥60 ml/min/1,73 m^2 sau ClCr ≥60 ml/min. Administrarea nu trebuie inițiată la pacienți cu RFGe <60 ml/min/1,73 m^2 sau ClCr <60 ml/min. La pacienții la care valorile RFGe scad în mod persistent sub 60 ml/min/1,73 m^2 sau ClCr sub 60 ml/min, doza trebule ajustată sau menținută la 10 mg o dată pe zi. Administrarea trebuie întreruptă la pacienții cu valori ale RFGe aflate persistent sub 45 ml/min/1,73 m^2 sau cu valori ale ClCr aflate persistent sub 45 ml/min. Nu trebuie utilizat la pacienții cu BRST sau cărora li se efectuează dializă. *IH* Nu este necesară ajustarea dozei la pacienți cu IH. Nu se recomandă utilizarea la pacienți cu IH severă. *Vârstnici* La pacienții cu vârsta de 75 ani și peste, trebuie avut în vedere un risc crescut de depleție volemică. Nu se recomandă începerea tratamentului cu empagliflozin la pacienții cu vârsta de 85 ani și peste. *Copii și adolescenți* La acest grup de pacienți, siguranța și eficacitatea administrării empagliflozin nu au fost încă stabilite. Nu sunt disponibile date. Mod de administrare Comprimatele pot fi administrate cu sau fără alimente, înghițite întregi cu apă. Dacă se omite o doză, aceasta trebuie administrată imediat ce pacientul își aduce aminte. În aceeași zi nu trebuie administrată o doză dublă. **Contraindicaţii:** Hipersensibilitate la substanța activă sau la oricare dintre excipienți. **Atenţionări şi precauţii speciale pentru utilizare:** Au fost raportate cazuri rare de CAD, inclusiv cazuri cu risc vital și cazuri letale la pacienți tratați cu inhibitori de SGLT2, inclusiv empagliflozin. Într-o serie de cazuri, natura afecțiunii a fost atipică, cu valori ale glicemiei mai mici de 14 mmol/l (250 mg/dl). Nu se cunoaște dacă CAD are o probabilitate mai mare de apariție în cazul utilizării dozelor mai mari de empagliflozin. Riscul de CAD trebuie luat în considerare în cazul apariției unor simptome nespecifice. Pacienții trebuie evaluați pentru depistarea CAD imediat ce apar aceste simptome, indiferent de valorile glicemiei. La pacienții unde se suspectează sau este diagnosticată CAD, tratamentul trebuie întrerupt imediat. Tratamentul trebuie întrerupt la pacienții care au fost spitalizați pentru intervenții chirurgicale majore sau afecțiuni medicale acute grave. La acești pacienți se recomandă monitorizarea cetonelor. Se preferă măsurarea valorii cetonelor din sânge, față de valoarea din urină. Tratamentul cu empagliflozin poate fi reînceput când valorile cetonelor s-au normalizat și starea pacientului a fost stabilizată. Înainte de a iniția tratamentul, trebuie luate în considerare antecedentele pacientului care pot predispune la CAD. Nu se recomandă reluarea tratamentului cu inhibitor de SGLT2 la pacienții cu antecedente de CAD dezvoltată în timpul tratamentului cu inhibitor de SGLT2 decât dacă a fost identificat și rezolvat un alt factor precipitant evident. Siguranța și eficacitatea empagliflozin la pacienții cu diabet de tip 1 nu a fost stabilită și empagliflozin nu trebuie utilizat pentru tratarea pacienților cu diabet de tip 1. *Monitorizarea funcției renale:* înainte de începerea tratamentului și periodic în timpul tratamentului, cel puțin anual; înainte de începerea tratamentului concomitent cu orice medicament care poate avea impact negativ asupra funcției renale. Leziuni hepatice Cazuri de leziuni hepatice au fost raportate atunci când s-a administrat empagliflozin în cadrul studiilor clinice. Nu s-a stabilit o relație de cauzalitate între empagliflozin și leziunea hepatică. Risc de depleție volemică Se impune prudență la pacienții la care scăderea tensiunii arteriale indusă de empagliflozin ar putea prezenta un risc. Până la corectarea pierderii de lichide, se va avea în vedere întreruperea temporară a tratamentului cu empagliflozin. Infecții ale căilor urinare În cadrul unor studii cumulate controlate cu placebo, frecvența infecțiilor căilor urinare raportate a fost similară la pacienții cărora li s-a administrat tratament cu empagliflozin 25 mg și placebo și mai mare la pacienții cărora li s-a administrat tratament cu empagliflozin 10 mg. La pacienții cărora li s-a administrat tratament cu empagliflozin s-au raportat ulterior punerii pe piață cazuri de infecții complicate ale căilor urinare. Întreruperea temporară a tratamentului cu empagliflozin trebuie avută în vedere la pacienții cu infecții complicate ale căilor urinare. Fasceită necrozantă care afectează perineul (gangrena Fournier). După punerea pe piață, s-au raportat cazuri de fasceită necrozantă care afectează perineul (afecțiune cunoscută și ca gangrena Fournier) la pacienți de ambele sexe tratați cu inhibitori de SGLT2. Este un eveniment rar, însă grav, care poate pune în pericol viața și care necesită intervenție chirurgicală urgentă și tratament cu antibiotice. În cazul în care există suspiciunea de gangrenă Fournier, se va întrerupe administrarea Jardiance® și se va institui imediat tratament (inclusiv antibiotice și debridare chirurgicală). IC Experiența la pacienți cu clasa NYHA I-II este limitată; nu există experiență în studiile clinice cu empagliflozin la pacienți cu clasa NYHA III-IV. În cadrul studiului EMPA-REG OUTCOME, la 10,1% dintre pacienți s-a raportat insuficiență cardiacă la momentul inițial. Scăderea numărului de decese de etiologie cardiovasculară la acești pacienți a fost similară cu cea înregistrată la populația de studiu totală. Evaluarea analizelor de laborator ale urinei Pacienții care urmează tratament cu Jardiance® vor avea glicozurie pozitivă. Lactoză Comprimatul conține lactoză. Pacienții cu afecțiuni ereditare rare de intoleranță la galactoză, deficit de lactază Lapp sau sindrom de malabsorbție a glucozei-galactozei nu trebuie să utilizeze acest medicament. **Fertilitatea, sarcina şi alăptarea:** Datele provenite din utilizarea empagliflozinului la gravide sunt inexistente. Este de preferat să se evite utilizarea Jardiance® în timpul sarcinii. Nu trebuie utilizat în timpul alăptării. Nu s-au efectuat studii privind efectele Jardiance® asupra fertilității la om. Studiile la animale nu au evidențiat efecte dăunătoare directe sau indirecte cu privire la fertilitate. **Efecte asupra capacităţii de a conduce vehicule şi de a folosi utilaje:** Jardiance® are influență mică asupra capacității de a conduce vehicule și de a folosi utilaje. Pacienții trebuie sfătuiți să ia măsuri de precauție pentru a evita hipoglicemia atunci când conduc vehicule și folosesc utilaje, în special atunci când Jardiance® este utilizat în asociere cu o sulfonilureе și/sau insulină. **Reacţii adverse (RA):** Siguranța administrării empagliflozinului a fost evaluată la un număr total de 15582 pacienți cu diabet zaharat de tip 2 incluși în studiile clinice. Reacția adversă raportată cel mai frecvent a fost hipoglicemia, atunci când se asociază cu sulfonilureе sau insulină. *Foarte frecvente:* hipoglicemie (atunci când se utilizează împreună cu sulfonilureе sau insulină). *RA frecvente:* candidoză vaginală, vulvovaginită, balanită și alte infecții genitale; infecții ale căilor urinare, sete, prurit, erupție cutanată tranzitorie, diureză crescută, creștere a lipidelor serice. *RA mai puțin frecvente:* urticarie, depleție volemică, disurie, creșterea creatininei sanguine/ scăderea ratei de filtrare glomerulară, creșterea hematocritului. *RA rare:* CAD; *cu frecvență necunoscută :* angioedem, fasceită necrozantă care afectează perineul (gangrena Fournier). **Data ultimei reînnoiri a autorizației:** 14/02/2019. **Data revizuirii textului**: 10/2019. **Jardiance® se eliberează pe bază de prescripție medicală PRF. Pentru informații suplimentare vă rugăm să consultați RCP complet, disponibil la cerere.**

Romanian Journal of Diabetes, Nutrition and Metabolic Diseases

Volume 27, issue4, October-December 2020

TABLE OF CONTENTS

ORIGINAL RESEARCH

Oral lichen planus and diabetes: A clinical study 303
Ioanina Părlătescu, Carmen Nicolae, Roxana Oancea, Cristian Funieru

Mitochondrial dysfunction of mesenchymal stem cells isolated from blood with type 2 diabetic patients 309
Svetlana Mykolaivna Gramatiuk, Irina Yuriivna Bagmut, Michael Ivanivich Sheremet, Julia Viktorivna Ivanova, Igor Leonidovich Kolisnik, Maria Sergiivna Gramatiuk, Dragos Cretoiu, Vitaliy Vasilyevich Maksymyuk, Volodimir Volodimirovich Tarabanchuk, Oleksandr Volodimirovich Lazaruk, Petro Vasilyevich Moroz

A 65-years-old woman with past history of diabetes mellitus experiences sudden acute onset of psychosis 316
Mansi Batra, SurajSandil,SumeetGupta, Harbir Kaur, Rohit Tiwari

The effects of exendin-4 on glucose homeostasis, pancreatic and duodenal homebox 1, and glucose transporter 2 gene expression disturbance induced by bisphenol A in male mice. The effects of exendin-4 on pancreatic gene expression 321
Akram Ahangarpour, Golshan Afshari, Seyyed Ali Mard, Ali Khodadadi, Mahmoud Hashemitabar

Glucose dependent insulinotropic polypeptide in impaired glucose tolerance and its association with insulin secretion and sensitivity 336
Marufa Akhter, Zebunnesa Zeba, Mamun Mia, Salima Akte,Rahelee Zinnat, Liaquat Ali

Study to evaluate the correlation between coagulation factor, glycemic control and the severity of diabetic foot ulcers among South Indian population: A case control study 342
Santhini Gopalakrishnan, Anuradha.G, Sumathy.S, Sandeep Unnikrishnan Dhanvarshini.S

Decrease of plasma TNF-α and CRP levels in response to post-exhaust resistance training and vitamin D supplementation in overweight healthy women. Exercise and vitamin D affect inflammation 349
Narges Kallantar, Hoseyn Fatolahi

The effect of Purslane and *Aquilaria malaccensis* on insulin-resistance and lipid peroxidation in High-fructose diet Rats 357
Samir Derouiche, Ouidad Degachi, Khaoula Gharbi

Serum levels of 8-hydroxy 2-deoxyguuanosine as a marker of DNA damage in healthy obese individuals 366
Abdelmarouf Hassan Mohieldein

Relationship of angiotensin converting enzyme (I/D) polymorphism (rs4646994) and ischemic heart disease in Iraqi patients with type 2 diabetes mellitus 372
Raghda N. Hemeed, Fadhil J. Al-Tu'ma, Dhafer A. F. Al-Koofee, and Ahmed H. Al-Mayali, Abdolmajid Ghasemian

Successful dietary intervention plan for Hashimoto's thyroiditis: A case study **381**
Nahla Subhi Al-Bayyari

Whey protein upregulates muscle insulin receptor tyrosine kinase and is comparable to vildagliptin as insulin-sensitizer **386**
Nahla El-Ashmawy, Eman Khedr, Hoda El-bahrawy, Enas El-Mokadem, Mariam Abo-Saif

Prescribing pattern of dipeptidyl peptidase 4 inhibitors and level of HBA1C target achievements among outpatients with type 2 diabetes mellitus in a Malaysian university teaching hospital **396**
Mohamed Hassan Elnaem, Mohamad Hafiz Hakeem Shamsuri, Nur Athirah Mohamad Aziz, Nurul Iman Alias, Rauha Farhana Hafizi, Nur Faezah Latif, Arina Norhazwani Rashid , Mohd Faris Aiman Mohamad Jalil , Mery Wei Ying Hu

Review

Inherited or acquired in hypertension and chronic kidney disease in diabetes mellitus patients **403**
Stoian Marilena, Dumitrache Ana Maria, Cîrciu Fivi, Stoica Victor, Radulian Gabriela

Emerging evidence on the association between COVID-19 and Type 2 Diabetes. Diabetes and COVID-19 **410**
Nasreem Bibi, Bahta Wara, Hana Morrissey, Patrick Ball

Dietary acid load: focus on body pH homeostasis and drug responses in type 2 diabetes **419**
Farid Berroukeche, Ismail Belkadi, Ouiam Halhali, Nassima Mokhtari-Soulimane, Hafida Merzouk

https://doi.org/10.46389/rjd-2020-1045
www.rjdnmd.org

Rom J Diabetes Nutr Metab Dis
2020; volume 27, issue 4, pages 303-308

Original Research

Oral lichen planus and diabetes: A clinical study

Ioanina Părlătescu[1], Carmen Nicolae[1], Roxana Oancea[2*], Cristian Funieru[3]

[1] Oral Pathology Department, Faculty of Dental Medicine, "Carol Davila" University of Medicine and Pharmacy, Bucharest, Romania
[2] Preventive Dentistry, Community and Oral Health Department, Faculty of Dental Medicine, "Victor Babeş" University of Medicine and Pharmacy, Timişoara, Romania
[3] Preventive Dentistry Department, Faculty of Dental Medicine, "Carol Davila" University of Medicine and Pharmacy, Bucharest, Romania

*Correspondence to: Roxana Oancea, Preventive, Community Dentistry and Oral Health Department, Splaiul Tudor Vladimirescu no. 14A, 300173 Timișoara, Romania. E-mail: roancea@umft.ro Phone: 40721335788

Received: 6 November 2020 / Accepted: 14 December 2020

Abstract

Background and Aims: Oral lichen planus (OLP) is an autoimmune chronic disease which is frequently related to some general diseases. The aim of this study is to analyze and compare the general features and clinical signs of OLP associated with diabetes mellitus. **Material and Method:** Twenty female patients suffering from OLP were enrolled in this study. They were examined and diagnosed following a clinical examination; and histologically, as well. The demographic and clinical features were collected from the medical charts. **Results:** In group 1 (OLP patients with diabetes) the oral complaints of pain and burning sensations were more frequently found compared to group 2 (OLP patients with nodiabetes). Diabetes patients showed an OLP clinical polymorphism – many clinical forms ($p<0.05$). The ulcerative type of oral lichen planus was found in group 1 only (4 from 10 cases). **Conclusions:** This study showed there is a higher frequency of oral complaints and ulcerative clinical form of OLP in diabetes patients.

Keywords: diabetes, lichen planus, oral lesions.

Background and Aims

Lichen planus is a chronic mucocutaneous disease with unknown etiology. It affects middle aged-adults, with a higher frequency in women. The oral lichen planus (OLP) is the mucosal analogous of cutaneous lichen planus and has an incidence estimated up to 2.2% [1].

The OLP pathogenesis is not completely known. Immune cells such as type T, macrophages, and Langerhan cells which induce apoptosis of oral keratinocytes are involved in the pathogenesis and progression of the disease [2].

The oral symptoms vary from reduced symptomatic lesions such as keratosis with white papules, reticular, or plaque-like pattern to painful and disturbing lesions as atrophy, erosion, ulcers, or bullae [3].

The OLP has some periods of flare-up and remission of the oral lesions and symptoms during its evolution. Complete healing is rare and all-life follow-up of these patients is recommended in order to avoid malignant transformation of OLP (rate of 1–2%) [3].

There are described some risk factors for OLP such as stress and anxiety. Moreover, the association between OLP and some general diseases such as diabetes mellitus, autoimmune thyroiditis, hepatitis C, and other immune disorders are frequently found [2].

Even the OLP is an autoimmune disease and the keratinocytes showing alterations of the membrane, enzymatic activity, and carbohydrate expression; it is also suggestive for a metabolic hormonal connection [4].

The association between OLP and diabetes was first mentioned in the 1960s and was confirmed later in many studies [5]. The incidence

© 2020 The Authors. Romanian Journal of Diabetes, Nutrition and Metabolic Diseases published by Sanatatea Press Group on behalf of the Romanian Society of Diabetes Nutrition and Metabolic Diseases. This is an open access article under the terms of the Creative Commons Attribution License (CC-BY-NC-ND 3.0).

of diabetes in OLP patients ranges from 14–85% according to the methodological line of every study [4].

A meta-analysis of OLP prevalence in diabetes patients determined a strong link between both diseases. Moreover, the risk of OLP in diabetes patients was found higher than in controls [6]. This higher risk supports the hypothesis of related pathogenesis between both diseases. The association between OLP and diabetes is considered by other authors as being full of controversy [5]. This is based on the idea that drugs used for diabetes can induce oral lichenoid lesions. The difference between OLP and oral lichenoid lesions is based on clinical and histological features. On the other hand, the Grinspan's syndrome described as OLP with diabetes mellitus and hypertension has also some controversies about its pathogenesis and the drug involvement during the metabolic conversion [7]. However, speaking about diabetes mellitus only, there are some oral cavity disorders frequently associated with diabetes and observed in common dental practice such as fungal infections, salivary hypofunction or periodontal diseases [8].

Since there are some inconsistent findings regarding OLP and diabetes in the literature, the present study aims to emphases some differences concerning OLP clinico-pathological aspects in diabetes patients compared to non-diabetic.

Material and Method

Study design and patients selection

This retrospective case-control study was developed on 20 female patients diagnosed with OLP who came in the clinic of Oral Pathology Discipline, Faculty of Dental Medicine, Carol Davila University of Medicine and Pharmacy, Bucharest, Romania. The patients were divided in two groups: 1 – OLP patients who also suffered from diabetes mellitus and 2 – OLP patients who did not have diabetes. The main selection criteria were the gender and age: female, above 50 years. The diagnosis of OLP was established by clinical and histological criteria in order to exclude other diseases [1].

The plasma glucose and HbA1 were measured and included in the diagnosis criteria for diabetes mellitus. In case of patients previously diagnosed with diabetes, the medical records, including laboratory tests, were analyzed.

Cases of Grinspan's syndrome were excluded from the present analysis. Moreover, patients with dysplastic changes or malignant lesions were also excluded.

All the patients included in this study signed an informed consent form, in accordance with the principles of Helsinki declaration. The study protocol was also approved by the Ethic Committee of Carol Davila University.

Data collection

The demographic data (age, gender), medical history, clinical and histo-pathological features were collected from the medical records. The personal data and the medical (and or surgical) history such as education level, smoking habit, main reason for the first medical appointment, and the onset details of the OLP lesions (e.g., the exact location on the oral mucosa, the clinical type of OLP) were also collected from the medical records.

The clinical forms of OLP were classified as follows: keratotic (reticular and/or plaques-like lesions), associated (keratotic and atrophic lesions), atrophic (mostly atrophic lesions), and ulcerative. All lesions were photographed and stored for later evaluation.

The mycological exams (exudate) of the oral lesion were also analyzed. The main features of OLP such as hyperkeratosis, basal cell vacuolization in the epithelium, and a specific band-like lymphocyte infiltrate in the corion were followed in the histopathology. One main role of histopathology in OLP was to detect the lack of epithelial dysplasia.

Statistical analysis

The data were entered into a computer using SPSS software, version 16 (SPSS Inc., Chicago, IL, USA). The correspondence analysis was performed in order to establish a possible

https://doi.org/10.46389/rjd-2020-1045
© 2020 The Authors

link between diabetes mellitus and OLP clinical forms.

Results

The age of patients (women only) selected for this study ranged from 50–73 years; mean age was 62.9 years.

The main characteristics of the entire sample (both study groups) are shown in table no. 1.

Patients from the first group said they felt burning sensation (six cases), pain (three cases), and one of them had no symptoms. In group 2, six patients reported no symptoms, two had burning sensation and two had pain.

One patient had diabetes type 1 and nine had diabetes types 2 group 1, eight of them being previously known with type 2 diabetes and two were diagnosed during OLP investigations. Six diabetes patients from group no. 1 were not under medical monitoring.

There were also some other general diseases in this study that were associated with OLP. The main disease was tiroiditis. In this study tiroiditis was present in three patients of group no. 1 and five patients from group no. 2.

The fungal test was positive for *Candida* in six patients in group no. 1 and in three cases in group no. 2.

The OLP lesions were found as follows: on both side (right and left) of buccal mucosa (90% in group no. 1 and 70% in group no. 2), tongue mucosa (50% in group 1 and 40% in group 2) and on gingiva (Fig 1).

The figure no. 2 shows number of cases of every OLP type. The ulcerative form was the most

Table 1: Characteristics of the 20 women enrolled in the study.

Characteristics	N
Age	
50-60 years	6
61-70 years	8
>71 years	6
Education*	
Medium	16
High	4
Location	
Urban	19
Rural	1
Diabetes	
Yes	10
No	10
Smoking habit	
Non-smokers	14
Former smokers	4
Smokers	2
Total	20

*Medium – at least high school; High – university diploma

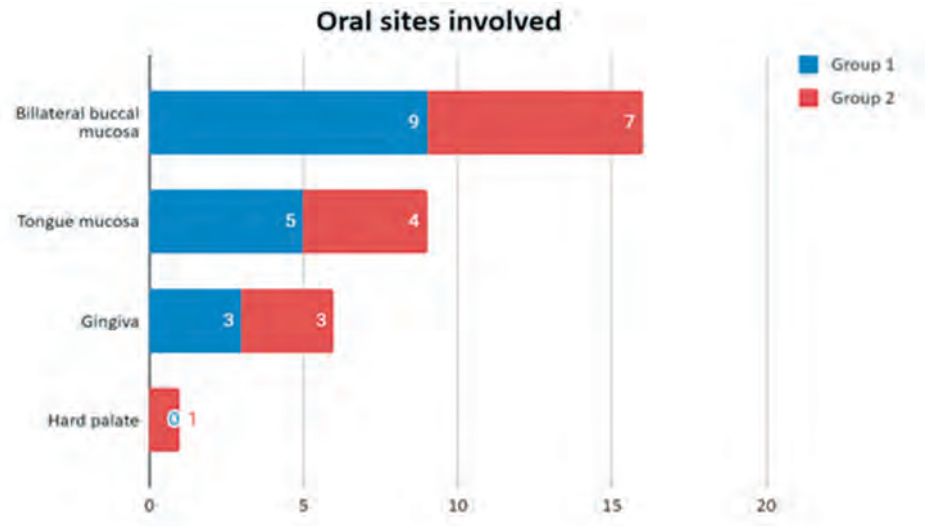


Figure 1: Oral sites involved in OLP lesions.

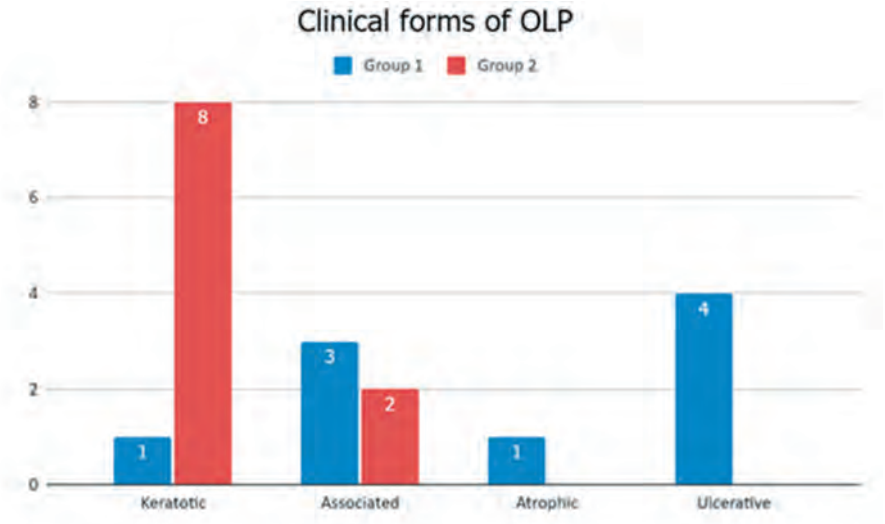


Figure 2: Clinical forms of OLP– number of cases.

common in group no. 1(40%) (Fig 3 a, b, c, d), followed by the associated type (30%). The keratotic form of OLP was the most frequently found in group no. 2(80%), (Fig 4 a, b, c).

Correspondence analysis was performed in order to establish a link between diabetes and OLP clinical forms. The polymorphism of OLP clinical forms (all four

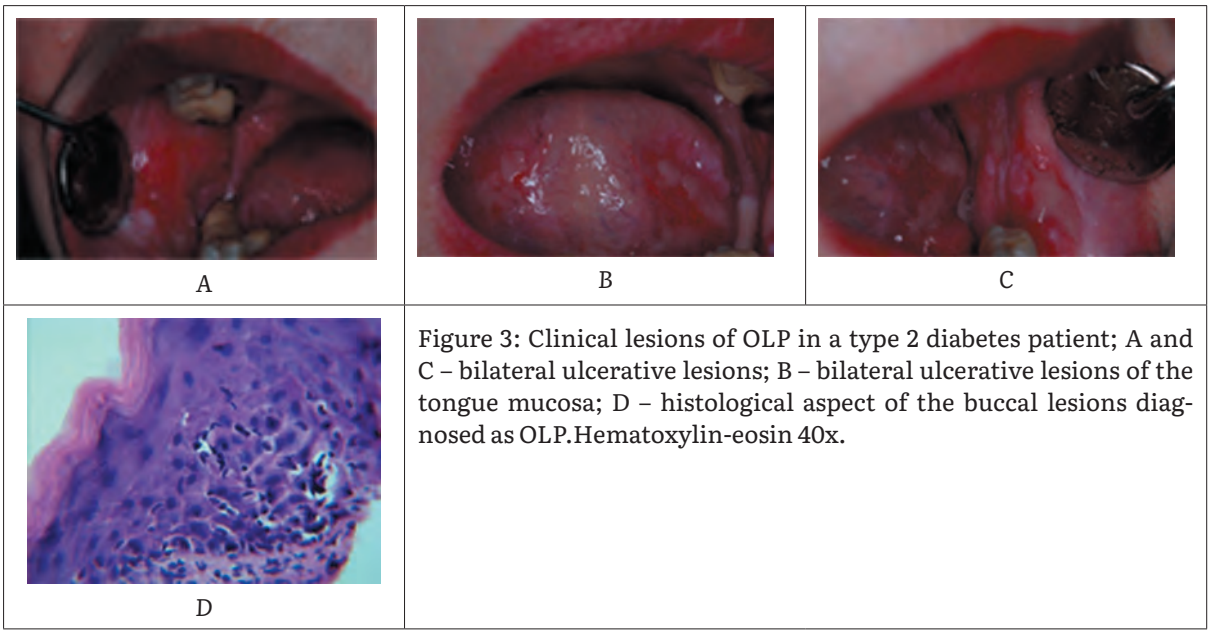


Figure 3: Clinical lesions of OLP in a type 2 diabetes patient; A and C – bilateral ulcerative lesions; B – bilateral ulcerative lesions of the tongue mucosa; D – histological aspect of the buccal lesions diagnosed as OLP.Hematoxylin-eosin 40x.

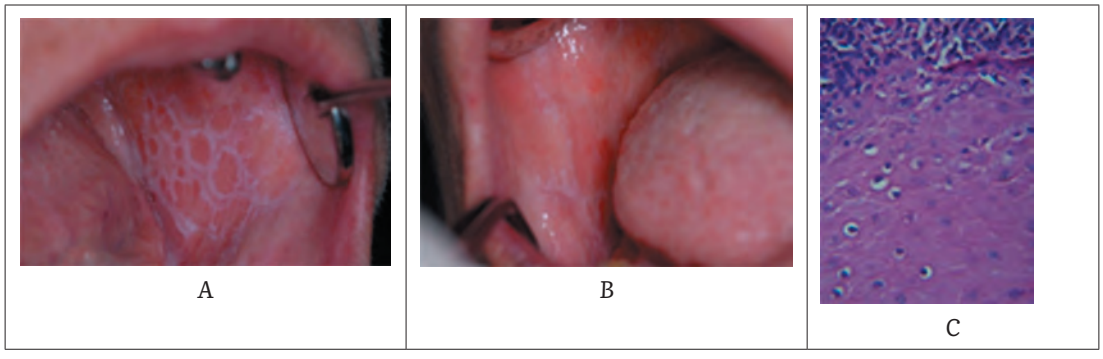


Figure 4: Clinical and histopathological images of OLP in a non-diabetes patient; A and B – bilateral keratotic lesions; C – Histopathological aspect of the OLP lesions. Hematoxylin-eosin 20x.

https://doi.org/10.46389/rjd-2020-1045
© 2020 The Authors

clinical forms) was present in diabetes patients ($p<0.05$).

Discussion

OLP is a chronic autoimmune disease with few periods of remission when the lesions tend to regress. The main (global) prevalence of OLP is near 1% with a geographical variation [9].

Only women were selected for this study since women suffer more frequently from OLP [10]. Moreover, the diabetes in association with OLP was reported more frequently in women [11], and, as it is well known, type 2 diabetes is more common in women [12].

The prevalence of OLP in diabetes patients is reported to be lower (under 4%) [12], but the prevalence of diabetes in OLP patients varies between 0.5% and 9.3% [6].

Most patients enrolled in our study were retired and/or had a low level of education.

In the present study the smoking habit was found in 10%, smoking being a risk factor for OLP in diabetes patients [6].

Almost all of our patients had type 2 diabetes. Some authors suggest that type 1 diabetes is more characteristic to be in association with OLP, as it is considered to be an autoimmune disease.

Many studies showed that oral signs/complains are very different [13]. These symptoms were found in most of our OLP patients with diabetes but only in few patients with OLP and no diabetes. The burning sensation of the oral mucosa was reported by some authors as the most frequent complaint in diabetes patients [8]. This is mainly connected with the peripheral neuropathic disturbances. The symptoms are very important because they determine the patient to ask for medical care and to receive an accurate diagnosis and a proper treatment. Another possible cause for these symptoms may be the oral candidiasis which can be frequently found in the OLP patients with diabetes compared to their counterparts. Moreover, the association between a reduced salivary flow rate and oral candidiasis are frequently noticed in diabetes patients [8].

The OLP clinical signs were frequently found in both groups on the buccal mucosa (bilateral) this being in line with other OLP studies [14] and considered to be typical for the OLP [3]. The tongue mucosa was the second most common site involved but no differences were found on this between both groups.

The ulcerative form of OLP was more frequently found in diabetes patients (four patients) when compared to non-diabetes patients. This finding is also noticed by Bagan et al. in their study developed on 72 patients where they reported a higher prevalence of atrophic and ulcerative lesions of OLP in patients with diabetes compared to patients with no diabetes [15]. A possible explanation for the presence of OLP ulcerative lesions in diabetes patients may be due to an increased period of healing which increase chances to be detected on time on the oral mucosa. Moreover, from six patients with uncontrolled diabetes, three of them presented ulcerative type of OLP.

The drug reaction is one of the important elements for the differential diagnosis of OLP in diabetes patients. It can appear on the oral mucosa as a side effect of medication for diabetes or antihypertensive medication. The clinical features show similarities with OLP but histopathology shows some important differences. In cases of lichenoid drug reaction, the inflammatory infiltrate of the connective layer has plasma cells and neutrophils [3]. The histopathology confirmed that all patients from our study suffered from OLP.

Conclusions

This study showed there is a higher frequency of oral complaints and ulcerative clinical forms of OLP in diabetes patients compared to non-diabetes. The frequency of oral sites involved was not influenced by the presence of diabetes. However, further investigation on a higher sample must be made.

Conflict of interest

The authors declare no conflict of interest.

© 2020 The Authors

References

1. Cheng YSL, Gould A, Kurago Z, Fantasia J, Muller S. Diagnosis of oral lichen planus: a position paper of the American Academy of Oral and Maxillofacial Pathology. *Oral surgery, oral medicine, oral pathology and oral radiology*.122(3): 332–354, 2016.
2. Hasan S, Ahmed S, Kiran R, Panigrahi R, Thachil JM, Saeed S. Oral lichen planus and associated comorbidities: An approach to holistic health. *Journal of Family Medicine and Primary Care*. 8(11): 3504, 2019.
3. Carrozzo M, Porter S, Mercadante V, Fedele S. Oral lichen planus: A disease or a spectrum of tissue reactions? Types, causes, diagnostic algorithms, prognosis, management strategies. *Periodontology 2000*. 80(1):105–125, 2019.
4. Zakaria ASM, Hossain MK, Bhuiyan MSI, Sultana A, Rahman M, Kulsom O. Disturbance in glucose metabolism in patients with lichen planus. *Bangabandhu Sheikh Mujib Medical University Journal*. 9(4):177–180, 2016.
5. National Elf Service [online], Retrieved from: https://www.nationalelfservice.net/dentistry/oral-medicine-and-pathology/oral-lichen-planus-diabetes-associated/ [Accessed 25 october 2020].
6. Mozaffari HR, Sharifi R, Sadeghi M. Prevalence of Oral Lichen Planus in Diabetes Mellitus: a Meta-Analysis Study. *Acta informatica medica: AIM: journal of the Society for Medical Informatics of Bosnia & Herzegovina: casopis Drustva za medicinsku informatiku BiH*. 24(6):390–393, 2016.
7. Manuel R, George R, Martin AM, Chacko A, Shalu S, Manuel S. Grinspan's Syndrome. *BMH Medical Journal*. 7(1):20–22, 2019.
8. Gandara BK, Morton TH. Non-periodontal oral manifestations of diabetes: a framework for medical care providers. *Diabetes Spectrum*. 24(4):199–205, 2011.
9. González-Moles MÁ, Warnakulasuriya S, González-Ruiz I, González-Ruiz L, Ayén A, Lenouvel D, Ruiz-Ávila I, Ramos-García P. Worldwide prevalence of oral lichen planus: A systematic review and meta-analysis. *Oral Dis*. 00:1–16, 2020.
10. Ingafou M, Leao JC, Porter SR, Scully C. Oral lichen planus: a retrospective study of 690 British patients. *Oral diseases*. 12(5):463–468, 2006.
11. Borghelli RF, Pettinari, IL, Chuchurru JA, Stirparo MA. Oral lichen planus in patients with diabetes: an epidemiologic study. *Oral surgery, oral medicine, oral pathology*. 75(4):498–500, 1993.
12. Petrou-Amerikanou C, Markopoulos AK, Belazi M, Karamitsos D, Papanayotou P. Prevalence of oral lichen planus in diabetes mellitus according to the type of diabetes. *Oral diseases*. 4(1):37–40, 1998.
13. Gorsky M, Raviv M, Moskona D, Laufer M, Bodner L. Clinical characteristics and treatment of patients with oral lichen planus in Israel. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*. 82:644–649, 1996.
14. Bagewadi A, Bhoweer AK. Oral lichen planus and its association with diabetes mellitus and hypertension. *Journal of Indian Academy of Oral Medicine and Radiology*. 23(5):300–303, 2011.
15. Bagan JV, Penarrocha M, Milian MA, Sanchis JM. Oral lichen planus and diabetes mellitus. A clinico-pathological study. *Bulletin du Groupement International pour la Recherche Scientifique en Stomatologie et Odontologie*. 36(1–2):3–6, 1993.

https://doi.org/10.46389/rjd-2020-1045
© 2020 The Authors

https://doi.org/10.46389/rjd-2020-1046
www.rjdnmd.org

Rom J Diabetes Nutr Metab Dis
2020; volume 27, issue 4, pages 309-315

Original Research

Mitochondrial dysfunction of mesenchymal stem cells isolated from blood with type 2 diabetic patients

Svetlana Mykolaivna Gramatiuk[1], Irina Yuriivna Bagmut[2], Michael Ivanivich Sheremet[3]*, Julia Viktorivna Ivanova[4], Igor Leonidovich Kolisnik[2], Maria Sergiivna Gramatiuk[1], Dragos Cretoiu[5], Vitaliy Vasilyevich Maksymyuk[3], Volodimir Volodimirovich Tarabanchuk[3], Oleksandr Volodimirovich Lazaruk[6], Petro Vasilyevich Moroz[3]

[1] Institute of Cellular Biorehabilitation, Ukraine Association of Biobank, Kharkiv 61000, Pushkinska str. 44, Ukraine
[2] Kharkiv Medical Academy of Postgraduate Education, Kharkiv, Ukraine
[3] Surgery Department No1, Bukovinian State Medical University[4], Ukraine
[4] State Institution «Zaytsev V.T. Institute of General and Urgent Surgery of National Academy of Medical Sciences of Ukraine», Kharkiv, Balakirev str. 1, Ukraine
[5] MD, Phd, Dr. MD – Centrul de excelenta M.F. al I.N.S.M.C., Bucuresti, Romania
[6] Department of Pathology (Pathology and Forensic Medicine), Bukovinian State Medical University, Ukraine

*Correspondence to: Michael Ivanivich Sheremet, Surgery Department No1 of Bukovinian State Medical University, Ukraine, Holovna str., 191, 58018 Chernivtsi, Ukraine. E-mail: mihayl71@gmail.com Phone: +0956064607

Received: 17 July 2020 / Accepted: 28 October 2020

Abstract

The Aim of the Study: is to focus on mitochondrial dysfunction, in the context of NASH, mitochondrial function in stem cells is likely to be impaired. **Materials and Methods:** Mesenchymal stem cell separated of peripheral blood from diabetes 2 type (DT2) patients was collected in the context of a clinical protocol authorized by the local Ethics Committee of Ukraine Association of Biobank (Ukraine), with a license from the Ministry of Health of Ukraine 04/10/2018 №1813 and 27/03/2019 №1231 by the national competent authority for biobank cord blood, cell and, tissue therapy. The study population (n = 96) was represented by diabetic patients from SI «ZIGUS NAMSU» in Kharkiv, Ukraine, and healthy volunteers. Patients were divided into two groups: group I consisted of patients with diabetes 2 type (DT2), group II - patients with DT2 complicated course of NASH (DT2 + NASH). The control group consisted of 25 conditionally healthy persons (men and women) of the same age. **Conclusion:** In the modern scientific space, various directions have been proposed in the diagnosis of metabolic syndrome and the treatment of D2T.

Introduction

Metabolic syndrome and finally diabetes 2 type (DT2) as a result of progressive obesity, insulin resistance, abnormal cholesterol or triglyceride levels are newfound problems in the current endocrinology. As reported by the International Diabetes Federation, in the entire world 382 million of adults (8.3%) are living with diabetes; the number is estimated to increase to 592 million in the next 20 years. More than 260 million people will be afflicted globally by 2022 [1–2].

Two main and interacting components determine the normal level of glucose in the blood, this is the reaction to insulin of skeletal muscle and liver, production of insulin by beta cells of the pancreas, under the influence of glucose. Therefore, two levels of defect have been identified, insulin resistance and progression to hyperglycemia. Modern data integrating these two hypotheses have a common, new direction inherent in the development of T2DM – mitochondrial dysfunction. [3–4].

Hepatic dysfunction in the form of nonalcoholic fatty liver disease (NAFLD) is commonly

© 2020 The Authors. Romanian Journal of Diabetes, Nutrition and Metabolic Diseases published by Sanatatea Press Group on behalf of the Romanian Society of Diabetes Nutrition and Metabolic Diseases. This is an open access article under the terms of the Creative Commons Attribution License (CC-BY-NC-ND 3.0).

observed in patients with T2DM [5–8]. Whether NAFLD is a cause or consequence of the diabetic pathology remains a topic of contention; however, the alterations in hepatic energy substrate metabolism and mitochondrial function in T2DM patients with NAFLD are well characterized. Decreased insulin sensitivity of the liver accompanied by increased hepatic fat storage are two such major metabolic changes found in the diabetic patients [9–11]. Mitochondria-intrinsic perturbations in obese, insulin-resistant patients with nonalcoholic steatohepatitis (NASH) include lower maximal respiration, increased mitochondrial uncoupling, and increased proton leak [12]. These findings are further strengthened by the observation of decreased ATP content and turnover in the T2DM liver [13–15].

Currently, significant progress has been made in the implementation of the anti-beta strategy, both at the clinical level and at the level of the national health system. The study of the metabolic syndrome and the search for an effective treatment for diabetes enable physicians to more deeply implement preclinical diagnostic correlates that allow the prevention of diabetes development at the primary level. Most effective in treatment includes e.g., glucagon-like peptide (GLP-1) mimetic, dipeptidyl-peptidase-4 (DPP-4) inhibitors, sodium glucose transporter-2 (SGLT2) inhibitors, but also surgical gastric correction, diet-related therapy, such as calorie restriction and finally mesenchymal stem cells application [5–8]. The other half of the 20th century has become a new stage in diabetes patients, so Friedenstein, and the knowledge about MSC is gaining biomedical significance in the natural sciences of biosystems. Unique cytophysiological properties of this stem cell population have led to developing a concept, in which their clinical application is consequently implemented [16–19].

The study focused on mitochondrial dysfunction was performed to unveil novel metabolism-related clues that may shed light on pathophysiology of T2D.

Materials and methods

The study population (n = 96) was represented by diabetic patients from SI «ZIGUS NAMSU» in Kharkiv, Ukraine, and healthy volunteers. All participants received and signed the informed consent, and the Ethical Committee approved the study. Mesenchymal stem cell separated of peripheral blood from T2DM patients was collected in the context of a clinical protocol authorized by the local Ethics Committee of Ukraine Association of Biobank (Ukraine), with a license from the Ministry of Health of Ukraine 04/10/2018 №1813 and 27/03/2019 №1231 by the national competent authority for biobank cord blood, cell, and tissue therapy.

The mean age of patients in the study was 61±6,3 years. Gender data: 56 (58,3%) – male, 40 (41,7%) – female. All studied patients had T2DM in anamnesis and met the World Health Organization diabetes and glucose intolerance criteria such as fasting plasma glucose(FPG) ≥126 mg/dL (7.0 mmol/L) or 2-hour oral glucose tolerance test (OGTT) plasma glucose ≥200 mg/dL (11.1 mmol/L), as well as polydipsia and polyuria in complex of symptoms.

Inclusion criteria: ability to read, understand, fill in and sign written informed consent; mentally healthy and ability to carry out the procedures of the study protocol; clinical history of type 2 diabetes mellitus (T2DM) according to the Expert Committee on the Diagnosis and classification of diabetes mellitus; the start of diseases of T2DM disease at ≥70 years of age; T2DM duration ≥5 and ≤15 years at the time of consent signature; level of C-peptide 0.28–2.1 ng/mL; HbA1c ≥ 7.1; patients must have been treated with SMT for minimum of five months prior to randomization. The injection insulin dose and metformin doses should be stable over the four months prior to randomization total insulin daily dose (TDD) at time of randomization should not exceed 1.0 units/day/kg; HbA1c≥7.1 and ≤9.0%.

Depending on the persistence of signs of hepatic dysfunction patients were divided in two groups: group I – patients with DT2, group II – patients with DT2 and NASH (DT2+NASH). The control group consisted of 25 healthy male and female comparable with age.

Exclusion criteria: insulin requirements of >1.0 units/day/kg; HbA1c >9.1%. (at the time of consent signature); C-reactive protein >2.85; arterial hypertension: SBP >160 mmHg or DBP >100 mmHg; evidence of renal dysfunction, serum

https://doi.org/10.46389/rjd-2020-1046
© 2020 The Authors

creatinine >1.6 mg/dl; proteinuria >290 mg/day; evidence of acute coronary syndrome in past 8 months and/or cardiovascular disease on physical exam.

For female exclusion criteria – pregnancy and/or presently breast-feeding, or unwillingness to use effective contraceptive measures for the duration of the study.

The exclusion criteria in men and women are- active infection including hepatitis C and B, HIV, or Tuberculosis. A history of coagulopathy and/or Factor V deficiency by INR>1.3, PTT>38, PT>13, or medical condition requiring long-term anticoagulant therapy.

Cell culture

All patients gave their written informed consent. MSCs were isolated from peripheral blood (PB) from method magnetic-separated in used automatic system AutoMACS, seeding 50,000 mononucleotide cells/cm^2 in RPMI (1x) +GlutaMAX medium (Gibco Life Technologies, Canada) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific), in CELLdisc™ a range of cell culture surfaces from 1,000 cm^2 up to 1 square meter.

The cultures were incubated at 37°C, 20% O_2, 5% CO_2 with used automatic system FibraStage (New Brunswick Scientific, USA). Medium changes were performed twice a week. Two weeks after initial seeding, primary MSC colonies were detached with a 10 min incubation at 37°C with Trypsine-EDTA 0.05% (Gibco Life Technologies, Canada) and replated at 4200 cells/cm2 in the same medium. Passage 6 MSCs were used for all experiments.

The characterization and standardization of MSCs are as plastic-adherent and spindle-shape, expression of antigen markers (CD73+, CD90+, CD105+, and CD45–, CD34–, CD14–, CD79–) on their surface and differentiation potential [19].

Live cell imaging

For identification of mitochondrial localization, cells were loaded with 200nMMitotracker Green FM in RPMI medium (no phenol red) in HBSS (Sigma-Aldrich). The images were obtained using a Zeiss LSM 400, a compact confocal scanning microscope equipped with 40X oil immersion objective.

The 488 nm was used to excite PKH26 Red Fluorescent Cell Linker (Kit for General Cell Membrane Labeling). Sigma's PKH26 dye is a red fluorescent cell labeling dye used for both in vitro/in vivo live cell imaging which was measured between 505 and 530 nm. All data presented were obtained from at least five coverslips and two to three different cell preparations.

For measurements of mitochondrial membrane potential ($\Delta\Psi m$), cells plated on 22 mm glass coverslips were loaded for 30 min at room temperature with 25 nMtetramethylrhodaminemethylester (TMRM; Invitrogen) in a HEPES buffered saline solution (HBSS) composed of 156 mM NaCl, three mMKCl, two mM MgSO4, 1.25 mM KH2PO4, two mM CaCl2, 10 mM glucose and 10 mM HEPES; pH adjusted to 7.35 with NaOH. The dye remained present in the media at the time of recording. The TMRM is used in the redistribution mode to assess $\Delta\Psi m$, and therefore a reduction in TMRM fluorescence represents mitochondrial depolarization.

For measurement of mitochondrial ROS production, cells were pre-incubated with Red DND-99 (L7528) and 75 nMMitoTracker® Green FM® for 10 min at room temperature measurements were produced using 580 nm excitation and emission above 600 nm.

Measurement of NADH/FAD redox index

The NADH auto-fluorescence was measured using an epifluorescence inverted microscope with a 20X fluorite objective. Excitation light at a wavelength of 350 nm was provided by a Xenon arc lamp, the beam passing through a monochromator (Life Technologies (ThermoFisher) EVOS XL Inverted Imaging Digital Microscope). Emitted fluorescence light was reflected through a 455 nm long-pass filter to a All EVOS® fluorescence imaging systems and the Countess® II FL Automated Cell Counter (Thermo Scientific EVOS Light Cube, DAPI) and digitized. Imaging data were collected

© 2020 The Authors

and analyzed using software from EVOS®. FAD auto-fluorescence was monitored using a Zeiss 710 VIS CLSM equipped with a META detection system and a 40x oil immersion objective. Excitation was using the 454 nm Argon laser line and fluorescence was measured from 505 to 550 nm. Illumination intensity was kept to a minimum (at 0.1–0.2% of laser output) to avoid phototoxicity and the pinhole set to give an optical slice of ~2 µm. FAD and NADH redox indexes and mitochondrial pools were estimated according the method described in Bartolome et al [8].

ATP assay method in MSCs

ATP was measured by luciferin-luciferase technique [29, 30] in which the amount of light generated by the reaction of ATP with recombinant luciferase is dependent on the ATP concentration. Sensitivity was augmented by addition of the D-luciferin to the luciferase. A 50 µl sample of MSCs, lysed with TCA 10% (tricoloroaceticacid) and neutralized with KOH 1 M and diluted with hepes buffer 100 mM pH 7.8 (1:64), injected into a cuvette containing 10 µlluciferin (sigma), 10 µl Mgso4, 10 µlluciferase (1 mg/ml). The peak light efflux from cuvette to which either known ATP standards or samples are added was determined using a luminometer (Sirius tube Luminometer, Berthold Detection System, Germany), a ATP standard curve was obtained on the day of each experiment.

Data and statistical analysis

Statistical tests: unpaired two-tailed Student's tests were performed using Medical Statistica 8.5 and Statistica 8.0 Microsoft Excel software (USA). Differences were considered statistically significant with p-value <0.01. Results are expressed as means ± standard error of the mean (SEM.).

Results

MSCs are taken from 96 diabetic patients who were diagnosed according to American Diabetes Association Guideline 2011 [35]. The baseline characteristic data are summarized in Table 1. In addition, MSCs of 25 healthy human volunteers with equal sex for all was sampled. Average ages for normal control individuals without medication and history of diabetes disease was 37.7 ± 4.9.

Changes in TMRM fluorescence showed a significant decrease in basal ΔΨm in patients with complicated TD2 MSC NASH, so the patients with TD2 MSC showed a significant decrease in mitochondrial membrane potential (p <0.001). Thus, in the TD2+NASH MSC group ΔΨm was reduced to 61.2 ± 3.2% (n = 36; p<0.001), while in the group of patients with diabetes mellitus this indicator was a bit higher – 75.4 ± 3.7% respectively (n = 50; p <0.001). Control group had ΔΨm MSC 85.2 ± 3.1% (n = 25).This data indicates possible mitochondrial impairment in mesenchymal stem cells in patients with TD2+NASH.

Table 1: Content ATP/ADP and mitochondrial membrane potential in MSCs group comparison, as shown in this table the difference between groups are significant (P-value <0.001)

Normal	Normal	Diabetes 2 type	Diabetes 2 type complicated course of NASH
N	25	50	36
BMI	25	37	41, 2
ATP/ADP	2.94 ± 0.11	5.03 ± 0.37	5.03 ± 0.37
HbA1C%	4.86 ± 0.13	4.09 ± 0.08	4.09 ± 0.08
FBS	84.05 ± 2.5	76 ± 0.94	76 ± 0.94
Mitochondrial (ΔΨm)	85.2 ± 3.1%	75.4 ± 3.7%	61.2 ± 3.2%

Note: P value < 0.001; BMI – Body Mass Index [weight/(height)^2] = kg/m^2; FBS – fasting blood sugar.

https://doi.org/10.46389/rjd-2020-1046
© 2020 The Authors

The results of study showed the expression of MSCs surface antigens CD with patients TD2 higher with accompanying NASH. So, we found that MSCs showed significantly decreased expression of CD90 by 96% that was also observed in patients TD2 higher with accompanying NASH.

So, in TD2 and NASH group, decreased CD105 surface antigens expression to 4% (n = 36; p<0.001) comparing to 81% (n = 25) of control group respectively that affected the properties stem cell. Based on the results obtained, we can make the assumption that progress of NASH with patients TD2 impairs the mitochondrial function of MSC (Fig. 1).

The total mitochondrial pool of NADH in MSCs with patients DT2 complicated course of NASH was also higher (p = 0.001) than in patients DT2 with level (60.3 ± 4.7%, n = 50) and control group with level (92.0 ± 5.5%, n = 25, (p<0.001)), indicating increased substrate availability for complex I in these cells (Fig. 2).

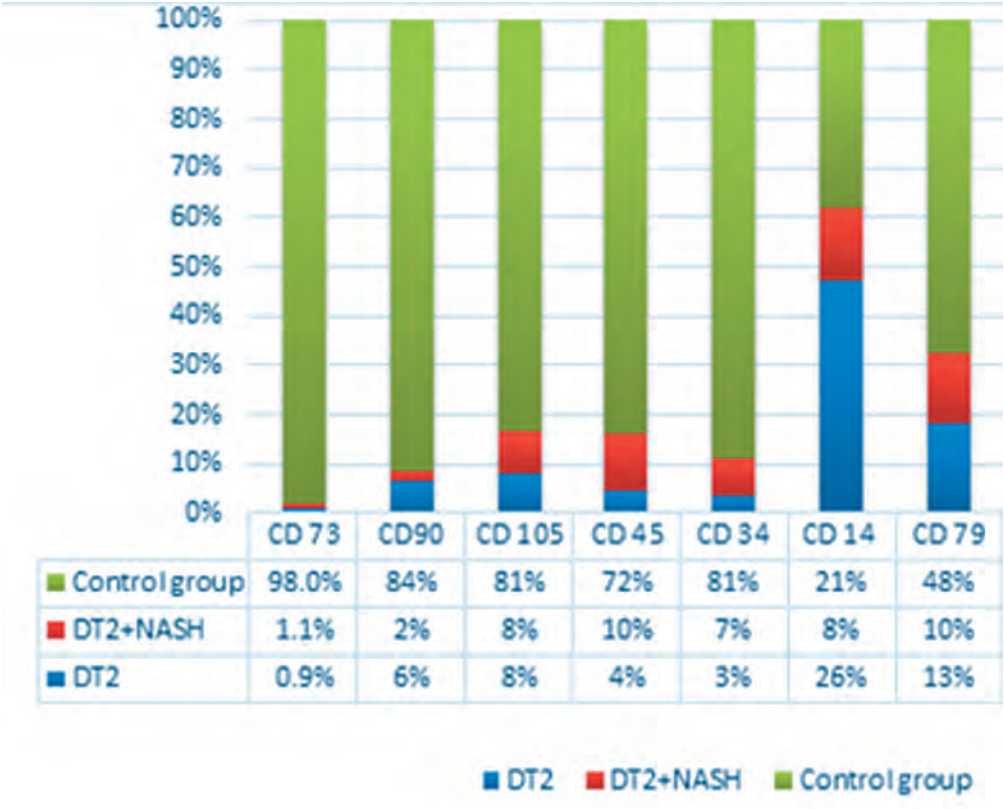
Quantification of the NADH redox index in MSCs from control and patient DT2, as measured with the mitochondria-specific probe established significant differences (p = 0.001) between the MSCs control group and group patients TD2 accompanying NASH, patients of TD2.

Discussion

The results of study showed that the decreased level of CD34 in progenitor stem cells and MSCs is associated with diabetes progression. In the healthy group patients MSCs in undifferentiated state in their positions (pools) remain stable. Despite "trigger" like pro-inflammatory cytokines IL-1 and/or IL-6, INF-α and growth factors VEGEF, TGEF are able to mobilize MSCs and induce their proliferation and homing the decrease in level MSC contributes impairment of all tissue regeneration [12–13].

Interestingly the by results of study MSCs cells in patients with TD2 were characterized by increased expression of CD44, working as an immune cell receptor involved.

Research conducted by Kodama and colleagues in experimental study revealed that CD44



	CD 73	CD90	CD 105	CD 45	CD 34	CD 14	CD 79
Control group	98.0%	84%	81%	72%	81%	21%	48%
DT2+NASH	1.1%	2%	8%	10%	7%	8%	10%
DT2	0.9%	6%	8%	4%	3%	26%	13%

Figure 1: The expression of antigen marker MSCs with patient's DT2 and control group.

© 2020 The Authors

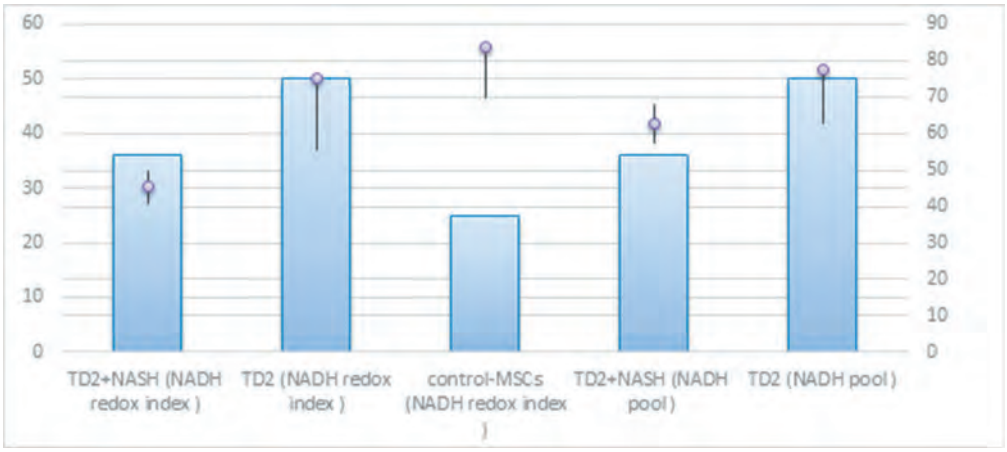


Figure 2: Quantification of the NADH redox index in MSCs from control and patient DT2.

was up regulated in white adipose tissue of obese, diabetic mice.

The same way, CD44 knockout mice fed a high fat diet, did not develop TD2 and/or obesity. The up regulation of CD44 led to migration and infiltration of activated immune cells, increasing the inflammation in adipose tissue; in addition, it was also confirmed that MS adipose tissue was enriched in macrophages secreting IL-1, IL-6 and TNF-alpha. Both obesity and TD2 strongly affect MSC morphology, including actin cytoskeleton organization [14–17].

The NASH affected the expression of patients TD2 MSCs surface antigens, as substantial reduction of CD90, CD105, and CD73 levels was observed. We found that TD2+NASH of MSCs showing significantly decreased expression of CD90 was also observed in patients suffering from TD2. The pro-inflammatory environment of adipose tissue negatively affects MSC stemness.

Stem cell metabolism is a great novel area for research. Changes in mechanisms of energy production in stem cell influence physiology and the course of the disease. Specifically, metabolism as well as changes in energy production has been associated with stem cell self-renewal and differentiation control [10–11].

Living cells are continuously exposed to the harmful effect of exogenous or endogenous reactive oxygen species (ROS). These highly reactive molecules, radicals and non-radicals, have the ability to capture electrons from molecules they come in contact with, including proteins and nucleic acids, leading in consequence to cell damage. Besides, oxidative stress may cause non-specific, post-translational protein modifications, leading to aggregate formation. Being the main source of ROS in cells mitochondrial respiratory complexes I and III are most susceptible to electron leakage, resulting in H_2O_2 formation. In addition, under certain conditions, the electron flux can be intensified, as in increased energetic demand during endurance exercises. On the other hand, mitochondrial function may decrease during aging or degenerative and metabolic diseases.

New evidences indicate the valuable role of mitochondrial dysfunction in the progression of NASH and T2D [18]. The conditions of hyperglycemia and high insulin tolerance lead to imbalances in ROS detoxication inside the cell, resulting in free oxygen radicals-mediated damage in both pathologies. It appears that the accumulation of defective mitochondria contributes to the reduced insulin secretion by β-cells [19-20].

It was shown that MSCs exhibited decreased mitochondrial membrane potential. The ATP/ADP rebalance in these cells was probably activated by excessive amount of ROS and damaged mitochondria, which led to nutrient and ATP deprivation.

Conclusion

The health of patients strongly affects the status of MSCs. Those cells isolated from patients with type 2 diabetes (TD2) or from patients

https://doi.org/10.46389/rjd-2020-1046
© 2020 The Authors

with TD2 accompanied by the NASH have the increased incidence of apoptosis, autophagy, accumulation of free radical molecules, and mitochondria deterioration. The mitochondrial membrane potential observed in these cells may be a protective mechanism that provides energy and building blocks to restore cellular homeostasis and control oxidative damage.

Based on presented data, our conclusion is that crucial metabolic aspects of TD2 are indeed recapitulated at the systemic level and perspective therapeutic application of MSC isolated from TD2 patients may be limited due to their dysfunctionality.

Conflict of Interest

The authors declare no conflict of interest.

References

1. Kornicka K, Houston J, Marycz K. Dysfunction of Mesenchymal Stem Cells Isolated from Metabolic Syndrome and Type 2 Diabetic Patients as Result of Oxidative Stress and Autophagy may Limit Their Potential Therapeutic Use. *Stem Cell Reviews and Reports*. 14:337–345, 2018.
2. IDF diabetes atlas - Home (n.d.). Retrieved from http://www.diabetesatlas.org/.
3. Rowe I A. Lessons from epidemiology: the burden of liver disease. *Dig Dis*. T.35:304–309, 2017.
4. Cholankeril G, Patel R, Khurana S, Satapathy S K. Hepatocellular carcinoma in non-alcoholic steatohepatitis: current knowledge and implications for management. *World J Hepatol*. 9:533–543, 2017.
5. Li Z, Li Y, Zhang HX, Guo J R, Lam CWK, Wang CY, Zhang W. Mitochondria-mediated pathogenesis and therapeutics for non-alcoholic fatty liver disease. *MolNutr Food Res*. 63:e1900043, 2019.
6. Einer S, Hohenester R, Wimmer L, Wottke R, Artmann S, et al. Mitochondrial adaptation in steatotic mice. *Mitochondrion*. 10.1016/j.mito.2017.08.015
7. Lim C Y, Jun D W, Jang S S, Cho W K, Chae J D, et al. Effects of carnitine on peripheral blood mitochondrial DNA copy number and liver function in non-alcoholic fatty liver disease Korean J. *Gastroenterol*. 55:384–389, 2019.
8. Bartolome F, Wu H C, Burchell V S, Preza E, Wray S, et al. Pathogenic VCP mutations induce mitochondrial uncoupling and reduced ATP levels. *Neuron*. 78(1):57–64.
9. Wang X, Du H, Shao S, Bo T, Yu C, et al. Cyclophilin D deficiency attenuates mitochondrial perturbation and ameliorates hepatic steatosis. *Hepatology*. 68:62–77, 2018.
10. Winkler S, Christ B. Treatment of NASH with Human Mesenchymal Stem Cells in the Immunodeficient Mouse/Animal Models for Stem Cell Therapy pp 51–56. https://doi.org/10.1007/978-1-4939-1453-1_5.
11. Plamena R, Mario A, Christopher B, Marta L, Dossenab, M V. Mitochondrial dysfunction in Parkinsonian mesenchymal stem cells impairs differentiation. *J. Redox Biology*. 14 474–484, 2018. https://doi.org/10.1016/j.redox.2017.10.016.
12. Hemeda H, Jakob M, Ludwig A K, Giebel B, Lang S et al. Interferon-gamma and tumor necrosis factor-alpha differentially affect cytokine expression and migration properties of mesenchymal stem cells. *Stem Cells and Development*. 19(5):693–706, 2010. https://doi.org/10.1089/scd.2009.0365.
13. Mansilla E, Marín G H, Drago H, Sturla F, Salas E et al. Bloodstream cells phenotypically identical to human mesenchymal bone marrow stem cells circulate in large amounts under the influence of acute large skin damage: new evidence for their use in regenerative medicine. Transplantation Proceedings. 38(3): 967–969, 2006. https://doi.org/10.1016/j.transproceed.2006.02.053.
14. Pérez L M, Bernal A, de Lucas B, San Martin N, Mastrangelo A García A, Gálvez B G. Altered metabolic and stemness capacity of adipose tissue-derived stem cells from obese mouse and human. *PloS One*. 10(4), 2015, e0123397. https://doi.org/10.1371/journal.pone.0123397.
15. Liu LF, Kodama K, Wei, K, Tolentino LL, Choi O, Engleman EG, McLaughlin T. The receptor CD44 is associated with systemic insulin resistance and proinflammatory macrophages in human adipose tissue. *Diabetologia*. 58(7): 1579–1586, 2015. https://doi.org/10.1007/s00125-015-3603-y.
16. Kodama K, Toda K, Morinaga S, Yamada S, Butte A J. Anti-CD44 antibody treatment lowers hyperglycemia and improves insulin resistance, adipose inflammation, and hepatic steatosis in diet-induced obese mice. *Diabetes*. 64(3): 867–875, 2015. https://doi.org/10.2337/db14-0149.
17. Kodama K, Horikoshi M, Toda K, Yamada, S, Hara, K et al. Expression-based genome-wide association study links the receptor CD44 in adipose tissue with type 2 diabetes. Proceedings of the National Academy of Sciences of the United States of America. 109(18): 7049–7054, 2012 https://doi.org/10.1073/pnas.1114513109.
18. Gonzalez C D, Lee M-S, Marchetti P, Pietropaolo M, Towns R, Vaccaro M I, Wiley J W (2011). *The emerging role of autophagy in the pathophysiology of diabetes mellitus*. *Autophagy*. 7(1): 2–11, 2011. https://doi.org/10.4161/auto.7.1.13044.
19. Jung H S, Chung K W, Won Kim J, Kim J, Komatsu M et al. Loss of autophagy diminishes pancreatic beta cell mass and function with resultant hyperglycemia. *Cell Metabolism*. 8(4): 318–324, 2008. https://doi.org/10.1016/j.cmet.2008.08.013.
20. Marsh B J, Soden C, Alarcón C, Wicksteed B L, Yaekura K, Costin A J, Rhodes C J. Regulated autophagy controls hormone content in secretory-deficient pancreatic endocrine beta-cells. *Molecular Endocrinology (Baltimore, Md.)*. 21(9): 2255–2269, 2007. https://doi.org/10.1210/me.2007-0077.

https://doi.org/10.46389/rjd-2020-1047
www.rjdnmd.org

Rom J Diabetes Nutr Metab Dis
2020; volume 27, issue 4, pages 316-320

Original Research

A 65-years-old woman with past history of diabetes mellitus experiences sudden acute onset of psychosis

Mansi Batra[1], Suraj Sandil[1], Sumeet Gupta[2]*, Harbir Kaur[3], Rohit Tiwari[1]

[1] **Department of Clinical Practice, Pharm D, M. M. College of Pharmacy, M. M. (Deemed to be University), Mullana, Ambala, (Haryana), India**
[2] **Department of Pharmacology, M. M. College of Pharmacy, M. M. (Deemed to be University), Mullana, Ambala, (Haryana), India**
[3] **Department of Medicine, M. M. Institute of Medical Science & Research, M. M. (Deemed to be University), Mullana, Ambala, (Haryana), India**

*Correspondence to: Dr. Sumeet Gupta, Professor, Department of Pharmacology, M. M. College of Pharmacy, M. M. (Deemed to be University), Mullana, (Ambala), Haryana, India. E-mail: sumeetgupta25@gmail.com Phone: +91 9872620252, +918059930156

Received: 14 April 2020 / Accepted: 28 July 2020

Abstract

Positive association between hypoglycemia and psychosis has been reported by many of the researchers and health care professionals. In our study case, we have reported a 65-year-old woman who diabetes mellitus with co morbidities for the past 20 years. For the last 10 days, she had experienced psychosis symptoms which showed abnormal behavior. As per the diagnosis by the physicians, inadequate insulin therapy may lead to hyperglycemic stage and psychosis symptoms. In order to prevent mental illness and as the best treatment for psychosis in diabetes patients, insulin therapy must be administered to the patient at the right time and needs regular follow up. In some of the cases it was also noted that, if the patients were left untreated they developed chronic mental illness with short term dementia.

Keywords: Abnormal behavior, Glucose disturbance, Mental health, Metabolic pathway.

Introduction

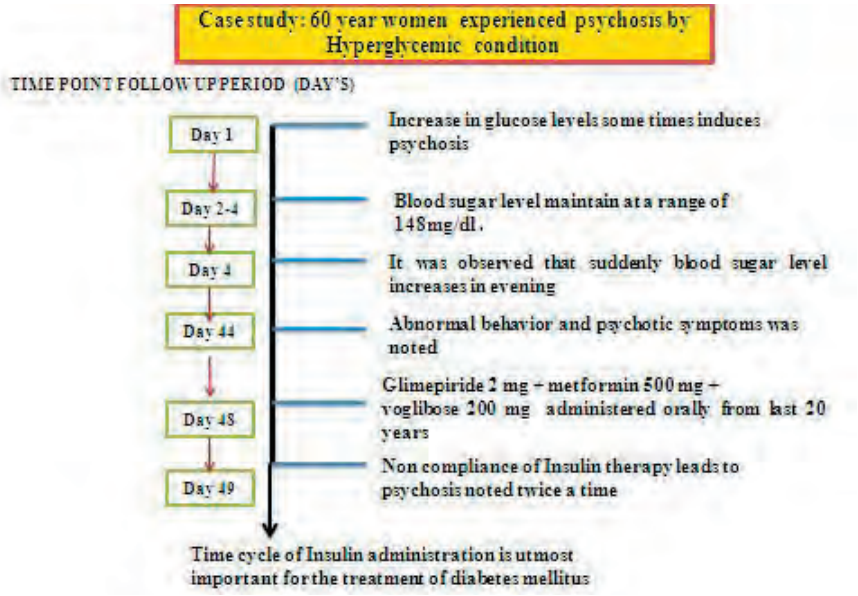
Diabetes mellitus is a metabolic disease characterized by high plasma glucose which if not controlled in time results in multiple micro and macro vascular complications. The prevalence of diabetes mellitus is increasing worldwide and affected 382 million people in 2013 and is expected to rise to 592 million by 2035 [1–2]. Long term left untreated it is recognized as the leading cause of end stage renal disease, non traumatic lower limb amputations, blindness, and a major cause of cardiovascular disease and stroke.

Mental illness and diabetes mellitus are closely linked. Type 2 diabetic patients with chronic hyperglycemia show abnormal behavior with aging. Depression and psychotic behavior are very well noticed in type 2 diabetes patients. The risk factor of schizophrenia or bipolar illness in diabetic patients is two to three times more than in the general population [3–7]. Diabetes mellitus and other metabolic risk factors may also change human behavior. However, association between diabetes mellitus and psychosis has not been reported in our studied population. We present the case of a female patient suffering from type 2 diabetes mellitus, who developed recurrent episodes of short lasting psychosis that are associated with metabolic syndrome.

Case Presentation

A 65-year-old female patient from rural area near Mullana was presented in the Emergency Department of the Hospital, having

© 2020 The Authors. Romanian Journal of Diabetes, Nutrition and Metabolic Diseases published by Sanatatea Press Group on behalf of the Romanian Society of Diabetes Nutrition and Metabolic Diseases. This is an open access article under the terms of the Creative Commons Attribution License (CC-BY-NC-ND 3.0).


Case study: 60 year women experienced psychosis by Hyperglycemic condition
TIME POINT FOLLOW UP PERIOD (DAY'S)
Day 1
Day 2-4
Day 4
Day 44
Day 48
Day 49
Increase in glucose levels some times induces psychosis
Blood sugar level maintain at a range of 148mg/dl.
It was observed that suddenly blood sugar level increases in evening
Abnormal behavior and psychotic symptoms was noted
Glimepiride 2 mg + metformin 500 mg + voglibose 200 mg administered orally from last 20 years
Non compliance of Insulin therapy leads to psychosis noted twice a time
Time cycle of Insulin administration is utmost important for the treatment of diabetes mellitus

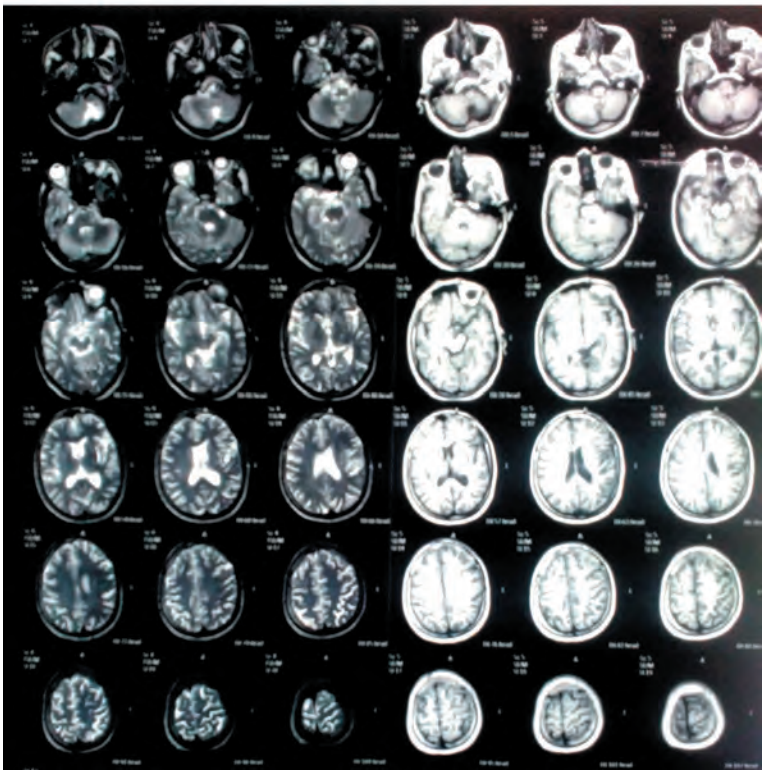


Figure 1: MRI of 65-years-old women showing normal brain

co-morbidies and acute onset psychotic illness for the past 10 days. The patient had a history of type 2 diabetes mellitus from last 20 years and hypertension since 10 years with coronary artery disease and congestive heart failure. She also had grade 4 diabetic nephropathy and with diabetic foot on left leg. Since last 10 days the patient was complaining of shortness of breath and swelling in bilateral lower limbs. From detailed case study – according to New York Heart Association guidelines, the patient had grade 4 dyspnoea with experience of Orthopenia, Paroxysmal Nocturnal Dyspnoea, and decreased urine output. Detailed exploration of the history revealed that she had experienced one to two similar psychotic episodes in the evening since last 10 days with each episode of being of 2 hrs duration. Over the last two months, she exhibited symptoms like agitation, abusive language, emotional labiality, smiling and muttering to herself, irrelevant talking,

© 2020 The Authors

poor self-care, decreased sleep, decreased appetite, and angry outbursts. During these episodes, there was no clouding of consciousness, disorientation, or disturbances in cognitive functions. After being admitted in hospital, we observed that psychosis episodes occurred every day at the same time during which, she exhibited symptoms like agitation, abusive language, irrelevant talking and angry outbursts with no other symptoms. At other times, she also experienced negative thoughts and false belief. Apart from psychotic illness, patient had fever, chest pain, palpitation and hypoglycemia four days back. The psychosis symptoms had no correlation with any substance abuse, fever, and infection. There was no family history of mental illness. The patient usually takes vegetarian diet and no wrong habit was observed.

Result

On day 1, the blood sugar level was maintained (148 mg/dl), the blood pressure level was found to be 160/90 mm Hg, respiratory rate was 34 per minute and the pulse rate was 96/minute. On day 2, blood sugar level was found to be 212 mg/dl. The physician's opinion was taken and insulin was administered as advised. Then, after three hours, blood sugar came down to the normal level. In the evening, nurse again checked the blood glucose level; it was 264 mg/dl. These observations were noted regularly for four days from the date of admission and we observed that, only in evening, the blood sugar level was high and psychosis episodes were also observed at the same time. She also had increased speech outbreak and complaint of threat to her life by the people around her. After every episode, the patient could not remember her activity which she had a few hours back. All other lab results regarding diagnosis including ketoacidosis were within normal range. Magnetic resonance imaging (MRI) revealed no abnormality in the brain. In general, pallor, cyanosis, and clubbing were absent. During physical examination, it was found that the patient had gangrene in left foot with big toe edema with cellulites. During CNS examination, the plantar was flexor and pupils show mild dilated. Drug management concerned regular use of oral tablet with combination of glimepiride 2 mg + metformin 500 mg + voglibose 200 mg prescribed by the physician and the patient was taking these medicine since last 20 years. Additionally, insulin was also started to normalize blood sugar levels as per requirement. Psychotic symptoms were resolved over the period of one week. No psychotropic drugs were prescribed. After two months following this episode, patient was again presented in OPD with similar psychotic symptoms and this again was due to non-compliance of drug therapy and insulin intake. The patient was made stable by slow administration of injection haloperidol over 5 min along with ascitalopram 10 mg and zolpidem 5 mg at bed time. Regular insulin was on sliding scale every six hourly. It was noted that, psychotic illness has temporal positive association between insulin therapy and rise in blood glucose levels in the normal range of 300 mg/dl. Following this, a final diagnosis was considered as diabetes mellitus induced psychosis and the patient was alerted by the physician about the importance of insulin and anti-diabetic drug.

Discussion

It is well established that there is a co-relationship between hypoglycemia and psychosis which sometimes leads to depression. Many of the studies trying to establish regarding abnormal behavior in diabetic mellitus patients induces psychosis [8–9]. We observed a very rare case in which hyperglycemia induced psychosis in a chronic patient which likely contributes to the lack of evidence based information on the mechanism of action in positive association manner. It may be possible that due to multiple pathogenesis, the metabolic disturbance may develop abnormal behavior especially in the hyperglycemic stage which is due to production of Advanced Glycation End products via stress radicals including polyol pathway, AGE pathway, thiamine metabolism pathway, hexamine pathway, PKC pathway and oxidative stress pathway [10–12]. Several studies reported about the control of hyperglycemia which is targeted at certain

 https://doi.org/10.46389/rjd-2020-1047 © 2020 The Authors

point in various mechanisms and this may be well defined to understand the exact association theory between psychosis and hyperglycemia.

Conclusion and limitations

From our case, it is clear to define, how important it is to treat a diabetic patient at the right time otherwise the patient may develop psychosis. There might be a correlationship between high blood sugar level and mental illness. In our present study, we noticed that, patients having inadequate control of blood sugar level had problem with symptoms of psychosis. Our case highlights the importance of insulin in diabetes patient with mental illness. Hyperglycemia exacerbates the psychosis symptoms and as suggested by the physicians, the patient was administered insulin and was relieved immediately from psychosis symptoms. This is the first case observed in our OPD which is well established by the follow up of the patients during the treatment. Proper medication and regular follow up may prevent mental illness in diabetes patients. Clinicians could be faced with problems due to some of the cognitive impairment symptoms as these symptoms could also be observed in many other underlying and undetected clinical conditions. As this patient is having multiple complications, we strictly recommended the use of latest a diagnostic tool like genomic study; it can help in the treatment of psychosis in acute diabetic patients. Further, we strictly did a follow-up of this patient for analyzing the exact cause and to observe any change in physical and mental behavior in the presence of drugs. Lastly, we conclude that this study will help further for analyzing different patients who are at risk for the development of psychosis.

Acknowledgment

The authors acknowledge the Head of Department of Medicine, M. M. Institute of Medical Science and Research, M. M. (Deemed to be University), Mullana, (Ambala), Haryana for providing case study and discuss regarding how important it is. The authors (SG) gratefully acknowledge the co-authors (MB, SS, HK, RT) and management for encouraging and providing the necessary facilities to regular follow up the patient. No funds granted from any sources for this study.

Statement of ethics

The study was assessed as per guidelines of World Medical Association, Declaration of Helsinki with prior permission from the patient during patient recruitment for the study conducted "Correlation ship between anti-hypertensive therapy and type-2 diabetes mellitus in population of Haryana" and it is approved by the IEC of M. M. (Deemed to be University)

Conflict of interest

The authors declare no conflict of interest.

Funding

Not received any funding from any sources

Author's contribution

Mansi Batra, Suraj Sandil contributes to conception, design, and acquisition of data, analysis and interpretation of data. Harbir kaur main lead for investigation and Rohit Tiwari supports the study. Sumeet Gupta drafts the manuscript and critically approved final manuscript.

References

1. Global report on diabetes world health organization 2016 http://apps.who.int/iris/bitstream/handle/10665/204871/9789241565257_eng.pdf;jsessionid=220C09C63024766119BCFF939774E111?sequence=1.
2. Pendlebury J, Holt RIG. Managing diabetes in people with severe mental illness. *J Diab Nur*. 14: 328–339, 2010.
3. Olsson E, Westman J, Hukic DS, Eriksson SV, Edman G, Boden R, et al. Diabetes and glucose disturbance in patients with psychosis in Sweden. *BMJ Open Diab Res & Care*. 3: e000120, 2015.

4. Surridge DH, Erdahl DL, Lawson JS, Donald MW, Monga TN, Bird CE, et al. Psychiatric aspects of diabetes mellitus. *Br J Psychiatry*. 145: 269–76, 1984.
5. Sahoo S, Mehra A, Grover S. Acute hyperglycemia associated with psychotic symptoms in a patient with type 1 diabetes mellitus: A case report. *Innov Clin Neurosci*. 13: 25–27, 2016.
6. Parab M, Mall G. A 55-year old obese woman with acute onset of psychosis. *Psychiatric Annals*. 45: 390–396, 2015.
7. Zrebiec J. Case study: cognitive impairment, depression and severe hypoglycemia. *Diab Spect*. 19: 212–215, 2006.
8. Balhara YPS. Diabetes and psychiatric disorders. *Ind J Endocrinol Metab*. 15: 274–283, 2011.
9. Tesfaye N, Seaquist ER. Neuroendocrine responses to hypoglycemia. *Ann N Y Acad Sci*. 1212: 12–28, 2010.
10. Stefano GB, Challenger S, Kream RM. Hyperglycemia-associated alterations in cellular signaling and dysregulated mitochondrial bioenergetics in human metabolic disorders. *Eur J Nutr*. 55: 2339–2345, 2016.
11. Gupta S, Jhawat V. Induction of type 2 diabetes mellitus with antihypertensive therapy: is there any role of alpha adducing, ACE and IRS-1 Gene?, *Value in Health Region* Iss 12c: 90–98, 2017.
12. Gupta S, Jhawat V. Quality by design (QbD) approach of pharmacogenomics in drug designing and formulation development for optimization of drug delivery systems. *J Contr Rel*. 245: 15–26, 2017.

https://doi.org/10.46389/rjd-2020-1047
© 2020 The Authors

https://doi.org/10.46389/rjd-2020-1048
www.rjdnmd.org

Rom J Diabetes Nutr Metab Dis
2020; volume 27, issue 4, pages 321-335

Original Research

The effects of exendin-4 on glucose homeostasis, pancreatic and duodenal homebox 1, and glucose transporter 2 gene expression disturbance induced by bisphenol A in male mice. The effects of exendin-4 on pancreatic gene expression

Akram Ahangarpour[1], Golshan Afshari[2]*, Seyyed Ali Mard[3], Ali Khodadadi[4], Mahmoud Hashemitabar[5]

[1] Department of Physiology, Health Research Institute, Diabetes Research Center, Faculty of Medical Sciences, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[2] Clinical Research Development Unit of Golestan Hospital, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[3] Physiology Research Center, Research Institute for Infectious Diseases of Digestive System and Department of Physiology, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[4] Cellular and Molecular Research Center, Department of Immunology science, Ahvaz Jundishapur University of Medical Sciences, Faculty of Medicine, Ahvaz, Iran
[5] Cellular and Molecular Research Center, Department of Anatomical Science, Ahvaz Jundishapur University of Medical Sciences, Faculty of Medicine, Ahvaz, Iran

*Correspondence to: Golshan Afshari, Golestan Hospital, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran, E-mail: golshanafshari59@gmail.com & Afshari.g@ajums.ac.ir, Phone: +989166004225, +986133743369, +986133764246.

Received: 9 March 2020 / Accepted: 25 May 2020

Abstract

Introduction: Bisphenol A (BPA) is a substance used in the packaging of food and beverages. It can bind to estrogen receptors with destructive consequences. Exendin-4 is a 39-amino acid peptide that bonds with GLP-1 receptors and stimulates insulin secretion. In this study, we aimed to explore the effect of exendin-4 on resolving BPA side effects. **Materials and Method:** We tested the effects of five concentrations of exendin-4 alone in combination with BPA on insulin secretion from isolated islets in in-vitro assay. Following in-vivo part of an examination, both BPA and exendin-4 were prescribed alone. Then, in combination for 20 days, next blood glucose, plasma insulin level, and Pdx1 and GLUT2 gene expression were examined. **Result:** Studies showed that BPA increased the blood glucose level, whereas exendin-4 was useful in removing these symptoms. Quantitative real-time polymerase chain reaction results (PCR) showed that BPA decreased the expression level of Pdx1 and GLUT2 genes in pancreatic tissue, whereas exendin-4 had a preventive role. In an in-vitro experiment, BPA increased the percentage of apoptotic cells, whereas exendin-4 restrained it. **Conclusion:** We observed evidence, such as a decrease in apoptosis in pancreatic islet cells and increase in Pdx1 and GLUT2 gene expression in the pancreas tissue. This can be an indication of the protective and supportive effects of exendin-4 on the pancreatic tissue, as well as the prevention of hyperglycemia in this assay.

Keywords: Bisphenol A, exendin-4, GLUT2, Pdx1.

Introduction

Diabetes is a prevalent disorder characterized by high blood glucose and is now-a-days increasingly recognized as a serious public health concern worldwide. Several studies have reported that the biological function of estrogen mediated through two separate nuclear receptors, including estrogen receptor α (ERα) and estrogen receptor β (ERβ), which are a part of the super family of nuclear receptors [1, 2]. There are various literature evidences which recognizes the

© 2020 The Authors. Romanian Journal of Diabetes, Nutrition and Metabolic Diseases published by Sanatatea Press Group on behalf of the Romanian Society of Diabetes Nutrition and Metabolic Diseases. This is an open access article under the terms of the Creative Commons Attribution License (CC-BY-NC-ND 3.0).

importance of estrogen receptors as an impressive molecule in glucose homeostasis, health, and metabolic disorders [3–5], so that ERα knockout mice gets afflicted with obesity and insulin resistance [6]. Nowadays, humans are inadvertently exposed to phenolic estrogen substances and endocrine disrupting chemicals (EDC), which lead to endocrine disorders [7] Bisphenol A (BPA), also known as a phenolic estrogen, is one of the most widely used substances in polycarbonate plastics, lining of canned foods and beverage bottles [8, 9]. BPA has been an object of research since 1970. As a common EDC, BPA intervenes with classical and non-classical estrogen receptors and through inappropriate activation of estrogen receptors, interferes in problems, such as type II diabetes and metabolic disorders [9, 10]. A primary concern is an easy displacement of BPA from canned food linings and plastic containers to their contents, and finally to the human body [11]. Recently, researchers have shown a direct relation between high urinary levels of BPA (>4.2 ng/mL) and type 2 diabetes]. What is known about BPA is based largely upon its ability to bind to estrogen receptors. Because estrogen receptors (ERα and ERβ) exist in beta cells, exposure to BPA is considered as a cause of glucose homeostasis disturbance [13]. Details of BPA effects on glucose metabolism are not well clarified, but several pathways, such as irregularity in insulin secretion through the mitochondria, cause damage and induce insulin resistance associated with oxidative stress attributed to BPA [14, 15]. In addition, BPA contributes to the development of hyperglycemia, down regulation of insulin receptors and insulin resistance [9, 15].

Glucagon-like peptide-1 (GLP-1) is a peptide hormone primarily released from intestinal L cells. GLP-1 plays a significant role in post-prandial insulin secretion, promoting insulin sensitivity, inhibiting glucagon secretion, and enhancing beta cell mass and insulin expression. GLP-1 has a short half-life and is rapidly degraded by dipeptidyl peptidase-4 (DPP-4) (16). The agonists of GLP-1R are new classes of drugs for type 2 diabetes. Exendin-4 is a 39-amino acid peptide that is extracted from the saliva of the Gila, a large lizard of North America, and has a 53% homology to GLP-1. The effects of exendin-4 on the human body are like those of GLP-1, though there are some differences, such as longer half-life and greater strength. Exendin-4 has been proven to enhance proliferating properties of pancreatic beta cells and prevents beta cell death in hyperglycemia and endoplasmic reticulum stress. Based on this evidence, exendin-4 is not an analogue of GLP-1 and, in fact, the joint activities of these two proteins in glucose regulation relate to their common organs in the pancreas [17, 18]. Various studies confirm the glucose homeostasis properties, beta cell mass protection, and neogenesis of pancreatic duct cells by exendin-4 [19–21]. Pdx1 is a transcription factor essential for pancreatic evolution and beta cells maturation. In addition to contributing to the survival of beta cells, Pdx1 also plays a role in enhancing insulin secretion, somatostatin, and beta-cell responses, so that any mutation leads to a variety of pancreatic-related diseases [22, 23]. Glut2 is an effective sensor and vector for glucose and is expressed on the plasma membrane of beta cells of the pancreas, liver, small intestine, and hypothalamus. Any disorder in the function of this protein has a negative effect on glucose homeostasis and disrupts the production of insulin from the beta cells [24].

A study in 2015 by J P. Tiano demonstrated significant synergistic effects of estrogen (E2) and GLP-1 on GSIS betterment. The study also reported that conjugated E2-GLP-1, by synergistic effects can play a role in preventing type 2 diabetes. One of mechanisms attributed to this process was inhibition of glucose production by liver [25]. Other similar studies have reported that simultaneous treatment with estrogen receptor agonists and GLP-1 improves insulin sensitivity in the liver tissue [26–28]. Since bisphenol is a substance that can bind to estrogen receptors and exendin-4 is a strong agonist of the GLP-1 receptors, therefore, in the present study, we have chosen to prescribe these two substances together.

In this study, an effective insulin tropic concentration of exendin-4 on isolated islets was selected and the islet cell apoptosis rate was evaluated in vitro. Further, the preventive effects of exendin-4 on glucose homeostasis and Pdx1 (pancreatic and duodenal homebox 1) and GLUT2 (glucose transporter 2) gene expression disturbed by BPA were evaluated.

https://doi.org/10.46389/rjd-2020-1048
© 2020 The Authors

Materials and Method

NMRI male mice with 25–30 g body weight was purchased from the animal house at the Ahvaz Jundishapur University of Medical Sciences. Animals were accommodated in BPA-free cages at 22°C ± 2°C, under a standard 12 h light/12 hour dark cycle with ad libitum access to food and tap water. All protocols executed were compatible with standards of animal care, demonstrated by the ethics commission (CMRC-96) of the Ahvaz Jundishapur University of Medical Sciences (Ahvaz, Iran). The anesthesia and euthanasia methods used in the current experiment were based on the method described by Carter et al. in 2009 [29], and on AVMA Guidelines for the Euthanasia of Animals, 2013 edition. We used male mice in the present study to elude the disconcerting effect of circulating estrogens in females. It should be noted that many studies on BPA have been performed on male laboratory specimens [30, 31], and cases involving female animals, mostly have been studied in the field of fertility and embryos [32, 33].

Our previous published study of BPA and exendin-4 had been focused on body weight, triglyceride, total cholesterol, LDL-cholesterol (LDLc), VLDL-cholesterol (VLDL–c) in plasma and also catalase (CAT), glutathione peroxidase (GPX), and superoxide dismutase (SOD) activity in pancreas tissue [34].

In-vitro protocol

About eight hours fasted intact mice were euthanized with an IP injection of ketamine (60 mg/kg) and xylazine (10 mg/kg) mixture. To expose all peritoneal cavity organs, the abdomen was cut surgically in a V shape. The common bile duct (CBD) near the small intestine junction was clamped, and 5 mL collagenase-p (Roche, Germany) was dissolved in 1.4 mg/mL concentration of Hank's balanced salt solution which was injected in the CBD junction of cystic and left hepatic ducts. After the pancreas started swelling, it was removed and allowed to digest at 37°C for 8–11 min. Further, digested tissue was centrifuged for 2 min at 1200 rpm, and the supernatant was discarded and all tubes were filled with Hank's solution. The rinse procedure was repeated 3–4 times. The islets were separated by hand picking under a stereo microscope and incubated at RPMI 1640+L-glutamine (Gibco Company, Germany) that was supplemented with 5 mM D glucose, 10% fetal bovine serum (FBS) (Gibco Company, Germany), 100 u/mL penicillin, and 100 µg/mL streptomycin (Gibco Company, Germany). Based on a previous experiment, 100 µg/L concentration of BPA (Sigma Aldrich Company, Germany) was selected for the in-vitro study [8, 31, 35].

Glucose stimulating insulin secretion

To select an effective concentration of exendin-4, first, five different concentrations of exendin-4 (2, 4, 8, 16 and 32 nM) were examined in the in-vitro condition (Sigma Aldrich Company, Germany. Cat No 141758-74-9, more than 97% purity). During the in-vitro experiment, the islets of different mice were pooled, and then divided into 12 groups so that each group has seven replicates each with five islets (Table 1). All groups were incubated at 37°C with 95% O_2 and 5% CO_2 for 24 h. Next, the islets in different groups were washed with Hank's solution, and all groups were incubated in three concentrations of glucose (2.8, 5.6, and 16.7 mM) for 60 min. Finally, the supernatant was collected and insulin secretion assessed using enzyme-linked immunosorbent assay (ELISA) method (Insulin ELISA Kit, Monobind, Inc, USA, code: 8525–300).

Apoptosis assay

After a dose response experiment of exendin-4 at the beginning of current study, the 4 nM concentration of exendin-4 determined as the selective insulinotropic dose in the in-vitro part and 4 nmol/kg/d of exendin-4 prescribe in the in-vivo experiment. Further glibenclamide is one of the most widely used drug as positive control group [36, 37] To evaluate islet cells wellness, isolated islets were pooled in culture medium and divided into five groups so that each group has

© 2020 The Authors

Table 1: In vitro groups for insulin secretion

Number	Description
1.	Control (culture media)
2.	2 nM exe-4 (media with 2 nM concentration of exendin4)
3.	4 nM exe-4 group (media with 4 nM concentration of exendin4)
4.	8 nM exe-4 group (media with 8 nM concentration of exendin4)
5.	16 nM exe-4 group (media with 16 nM concentration of exendin-4)
6.	32 nM exe-4 group (media with 32 nM concentration of exendin-4)
7.	BPA group (media with 100 µg/l concentration of BPA)
8.	BPA + 2nM exe-4 group (media with 2 nM concentration of exendin-4 and 100 µg/L concentration of BPA)
9.	BPA + 4nM exe-4 group (media with 4 nM concentration of exendin-4 and 100 µg/L concentration of BPA)
10.	BPA + 8 nM exe-4 group (media with 8 nM concentration of exendin-4 and 100 µg/L concentration of BPA)
11.	BPA + 16nM exe-4 group (media with 16 nM concentration of exendin-4 and 100 µg/L concentration of BPA)
12.	BPA + 32nM exe-4 group (media with 32 nM concentration of exendin-4 and 100 µg/L concentration of BPA)

four replicates with 10 islet: 1) control group (culture media), 2) BPA group (culture media with 100 µg/L concentration of BPA [9, 31, 35]), 3) the BPA + glibenclamide (Gb) group (culture media with 3 mg/L concentration of Gb and 100 µg/L concentration of BPA), 4) the BPA + exe-4 group (culture media with 4 nM concentration of exendin-4 and 100 µg/L concentration of BPA), 5) the exe-4 group (culture media with 4 nM concentration of exendin-4). Further, 11 mM of glucose concentration was established for all groups because the minimum rate of apoptosis and maximum viability rate of rodent islet cell occurs at this concentration [38]. Islets were incubated at 37 ˚C with 95% O_2 and 5% CO_2 for 48 hour. The medium was replaced with a fresh one every 24 hour. After this period, the medium was removed and the cells washed with phosphate buffer saline. Then trypsin was used for intracellular junction destruction. Next, the percentages of apoptosis of islet cells were measured using Annexin V Apoptosis detection kit FITC (According to the instructions, eBioscience, Cat 88-8005) and flow cytometry. Finally, the data were analyzed using the Win Med 2.9 software.

In-vivo protocol

In total, 40 adults, 2.5–3 months aged NMRI male mice were acclimatized for one week in a standard room. The mice were divided into five experimental groups (Table 2). The control group received solvent every day, BPA group received 100 µg/kg/d BPA for 20 days, the BPA + Gb group received 100 µg/kg/d BPA for 10 days and co-administration of 3 mg/kg/d Gb and BPA in the last 10 days, BPA + exendin-4 group received 100 µg/kg/d BPA for 10 days and co-administration of 4 nmol/kg/d exendin-4 and BPA in the last 10 days, and exendin-4 group received 4 nmol/kg/d exendin-4 for 20 days. BPA solvent was ethyl alcohol with a final concentration of 0.1%, exendin-4 and the glibenclamide solvent was distilled water. To induce disturbance, BPA was injected subcutaneously in order to ensure better absorption and, according to previous studies, there was no significant difference in plasma levels of BPA with different prescribing methods [39]. Exendin-4 was administered by IP injection, and glibenclamide was administered orally once daily. Our treatment period was chosen based on our earlier studies [40].

https://doi.org/10.46389/rjd-2020-1048
© 2020 The Authors

Table 2: In vivo experimental groups

	Groups/Days	Days 1–10	Days 11–20
1	Control	Solvent	Solvent
2	BPA	BPA	BPA
3	BPA + glibenclamide	BPA	BPA + glibenclamide
4	BPA + exendin-4	BPA	BPA + exendin-4
5	Exendin-4	Exendin-4	Exendin-4

Blood collection and biochemical assay

After treatment duration, mice were anesthetized with intraperitoneal injections of ketamine (60–80 mg/kg) and xylazine (10 mg/kg) mixture. The blood glucose level was measured through blood sampling from the tail by glucometer after 8–9 hours of fasting. Next, a cardiac puncture was performed to get more blood, and then plasma was separated by centrifugation (4000 rpm, 10–12 min). Enzyme-linked immunosorbent assay kit (Insulin ELISA Kit, Monobind, Inc, USA, code: 8525–300) was used to assay the plasma insulin level. The within-assay and between-assay coefficients of variation were 4.3% and 9.5%, respectively. Blood samples from the heart and pancreas tissue were rapidly removed and frozen in liquid nitrogen.

Quantitative real-time polymerase chain reaction (PCR)

RNA was extracted from homogenized pancreas tissues of five different groups using Qiagen RNeasy Plus Mini Kit (Cat 74134 USA); Thermo Scientific kit (K1621 USA) was used for cDNA synthesis. To perform quantitative real-time PCR, Thermo Scientific Maxima SYBR Green/ROX qPCR Master Mix2X (K0221 USA) was used. For Pdx1, GLUT2, and β-actin mRNA expression, the following primer sequence was used, according to the Suzuki study (Table 3) [41]: Quantitative real-time PCR (95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing and extension at 60°C for 30 s) performed with ABI plus (7000 PCR instrument, Applied Biosystems US). The level of these gene expressions normalized to β-actin as a housekeeping gene. The results were based on the $2^{-\Delta\Delta CT}$ method and relative quantification. The mean expression value of the control group was considered as one.

Statistical analysis

Before statistical analysis, normal distribution and homogeneity of the variances were evaluated using Levene's test, then by using one-way analysis of variance followed by Tukey's as post hoc test and was presented in figures as mean ± SEM. Differences were considered statistically significant at P values <0.05.

Table 3: Primers used for real-time PCR

Gene	Primer	Sequence (5′-3′)	PCR length
Pdx1	Pdx1 Forward	CCG AGA GAC ACA TCA AAA TCT GG	80 bp
	Pdx1 Reverse	CCC GCT ACT ACG TTT CTT ATC TTC C	
GLUT2	GLUT2 Forward	TTG ACT GGA GCC CTC TTG ATG	73 bp
	GLUT2 Reverse	CAC TTC GTC CAG CAA TGA TGA	
β-ACTIN	β-ACTIN Forward	GGC CAA CCG TGA AAA GAT GA	79 bp
	β-ACTIN Reverse	CAC AGC CTG GAT GGC TAC GT	

© 2020 The Authors

Result and Discussion

Results

In-vitro results

The effect of different concentration of exendin-4 alone and in combination with BPA on glucose-stimulated insulin secretion (GSIS), in vitro

Exendin-4 in five different concentrations and three various doses of glucose (Table 1) increased the insulin secretion from isolated islets, but the highest values were at 4 nM concentration ($p<0.001$ in comparison with the control group, Table 4). At higher values of exendin-4 (>4 nM of exendin-4), insulin secretion from isolated islets was less. BPA reduced by approximately 31% the amount of insulin secretion from isolated islets in comparison with the control group and this reduction, only at 16.7 mM concentration of glucose was significant ($p<0.01$). The concomitant use of exendin-4 with BPA elevated insulin secretion and could increase the level of insulin secretion to the same level of the control group (Table 4).

Islet cells apoptosis

Apoptosis will be detected initially by staining the cells with Annexin V and propidium Iodide solution followed by flow cytometry analysis. In the chart provided by flow cytometry (dot plot chart), the upper left quadrant displayed necrosis, whereas the left lower quadrant displayed the healthy cells. The upper right quadrant revealed dead cells and the lower right quadrant displayed early stage of apoptosis of cells, which in the current experiment are reported as apoptotic cell (Fig. 2). [42]. The evaluation of apoptosis results showed that in the BPA group, the percentage of apoptosis in islet cells was significantly increased ($p<0.001$) and the percentage of healthy cells were obviously decreased ($p<0.001$), compared to the control group. As expected, there are similarities between control group and exendin-4 group in the percentage of apoptosis, so that exendin-4 group showed the lowest percentage of apoptosis. The study of the percentage of normal cells by flow cytometry revealed that BPA significantly reduced the level of normal cells, and in co-administration of BPA and exendin-4, reduction in normal cell count did not occur (Fig. 1).

In-vivo results

Fasting blood glucose (FBG)

BPA increased fasting blood glucose compared with control ($p < 0.001$), and co-administration of exendin-4 with BPA modified blood glucose levels. The results of BPA+ exendin-4 group were similar to those of the BPA + Gb group, and using exendin-4 alone in a 20-day period had no effect on fasting blood glucose (Fig. 3).

Plasma insulin level

Plasma insulin level in the BPA group significantly reduced ($p<0.01$) in comparison with the control group, and co-administration of BPA with exendin-4 significantly increased the level of insulin in comparison with the control group ($p<0.01$) and BPA ($p<0.001$) group (Fig. 4).

Pdx1 and GLUT2 gene expression

Quantitative real-time PCR results of pancreatic tissue showed that BPA significantly decreased ($p<0.05$) the level of Pdx1/β-Actin expression in comparison with the control group. Conversely, in the group where BPA was administered in combination with exendin-4, the expression level of Pdx1/β-Actin showed a significant increase ($p<0.05$) in comparison with the BPA group. Moreover, in the exendin-4 group, the expression level of Pdx1/β-Actin increased significantly ($p<0.001$) in comparison with the control group (Fig. 5). QRTPCR results of GLUT2/β-Actin gene expression in different groups showed that BPA significantly ($p<0.01$) reduces the level of gene expression. The co-administration of BPA

https://doi.org/10.46389/rjd-2020-1048
© 2020 The Authors

© 2020 The Authors

Table 4: In vitro insulin secretion

	control	2nM exe-4	4nM exe-4	8nM exe-4	16nM exe-4	32nM exe-4	BPA	BPA+2nM exe-4	BPA+4nM exe-4	BPA+8nM exe-4	BPA+16nM exe-4	BPA+32nM exe-4
2.8mM Glucose	0.09 ± 0.004	0.11 ± 0.008	0.12 ± 0.007	0.12 ± 0.015	0.12 ± 0.025*	0.12 ± 0.013*	0.07 ± 0.005	0.07 ± 0.007	0.08 ± 0.01	0.12 ± 0.014*#	0.09 ± 0.007	0.07 ± 0.014
5.6mM Glucose	0.11 ± 0.004	0.11 ± 0.007	0.16 ± 0.01***	0.12 ± 0.008*	0.12 ± 0.014*	0.12 ± 0.009*	0.08 ± 0.002	0.09 ± 0.005	0.11 ± 0.023#	0.11 ± 0.004#	0.09 ± 0.013	0.09 ± 0.004
16.7mM glucose	0.13 ± 0.004	0.13 ± 0.011	0.2 ± 0.32***	0.12 ± 0.007	0.12 ± 0.009	0.12 ± 0.01	0.08 ± 0.005**	0.10 ± 0.007	0.12 ± 0.018#	0.11 ± 0.003#	0.10 ± 0.014	0.10 ± 0.004

Insulin secretion ng/ml/islet/60 min from isolated islet after 24 hour incubation in different concentration of exendin-4 and 100 µg/L BPA and then incubation in three doses of glucose for 60 min. All groups compared with its relative control group in terms glucose concentration. *p<0.05, **p<0.01, ***p<0.001 vs. control group. #p<0.05 vs. BPA group. Data are expressed as mean ± SEM.

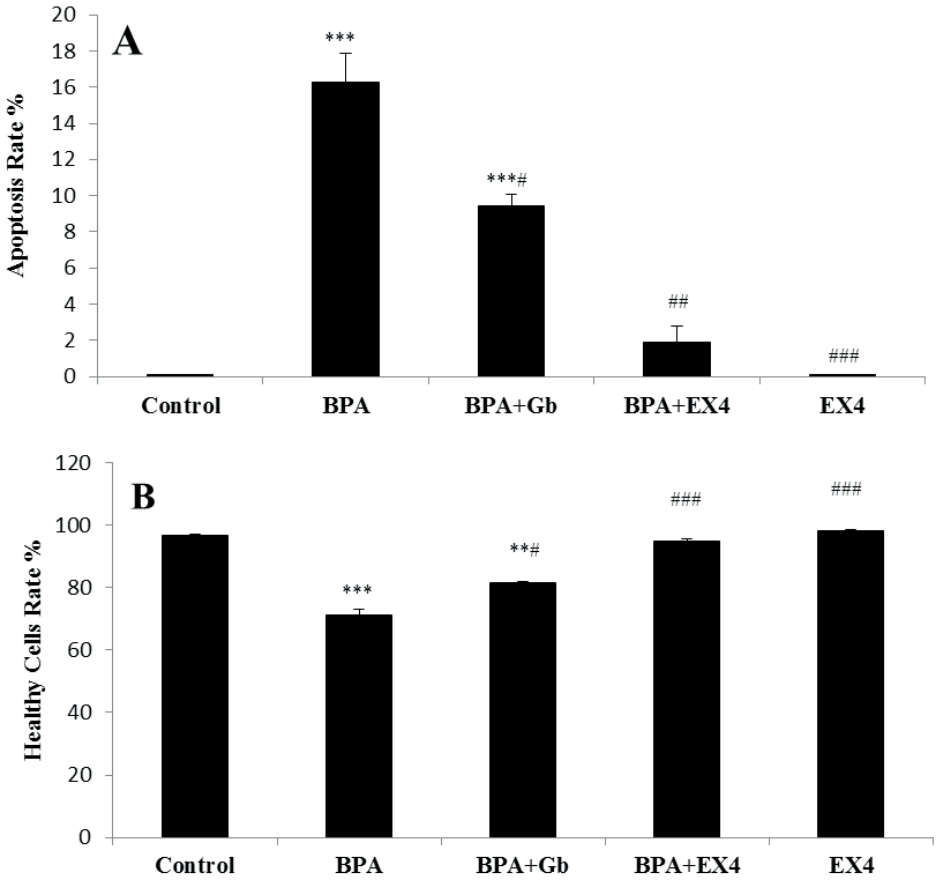


Figure 1: *Effect of BPA and exendin-4, alone and together, on pancreatic islet cell Apoptosis* (A) and healthy (B) after 48 h incubation of intact islets in five in vitro groups. **p<0.01, *** p<0.001 vs. control. #p<0.05, ##p<0.01, ### p<0.001 vs. BPA group. Data is expressed as mean ± SEM, based on the percentage provided by flow cytometry (n = 4).

and exendin-4 compensates for this reduction similar to the control group. In the exendin-4 group, there was a significant (p<0.001) increase in GLUT2/β-Actin gene expression than in the control and BPA groups (Fig. 6).

Discussion

This study aimed to evaluate the effects of exendin-4 on glucose homeostasis and gene expression complicated by BPA. This paper first reported about exendin-4 effects on GSIS improvement, and also highest values of insulin secretion at doses of 5.6 and 16.7 mM of glucose and the finding is in agreement with Niu B et al. findings [43]. Consistent with our study, Padmasekar et al. observed that exendin-4 increased insulin secretion from INS-1E cells and isolated islets of mouse in a dose-dependent manner [44]. Several studies have shown that phenolic estrogen induces morphological changes in isolated islets and impairs the amount and content of insulin secretion [45–47]. In addition, BPA disrupts the endocrine system through interaction with the ER and through other pathways, including those of oxidative stress [40, 46, 48], insulin signaling disturbance [49], and beta cell apoptosis [13, 40]. A recent literature has emerged that the effect of BPA on insulin secretion has an inverse U shape in a dose-dependent manner. At doses as low as 100 pM–1 nM of BPA, insulin secretion increases, but higher doses of exendin-4 induced decrease in the content and secretion of insulin from islets [50]. It is interesting to note that, an increase in the amount of insulin released in response to glucose stimulation in the presence of BPA, as seen in some literature, is due to the depletion of high content of insulin in beta cells [51]. As mentioned in the prior studies, just because there is no significant reduction in insulin secretion in BPA group, we cannot ignore its

https://doi.org/10.46389/rjd-2020-1048
© 2020 The Authors

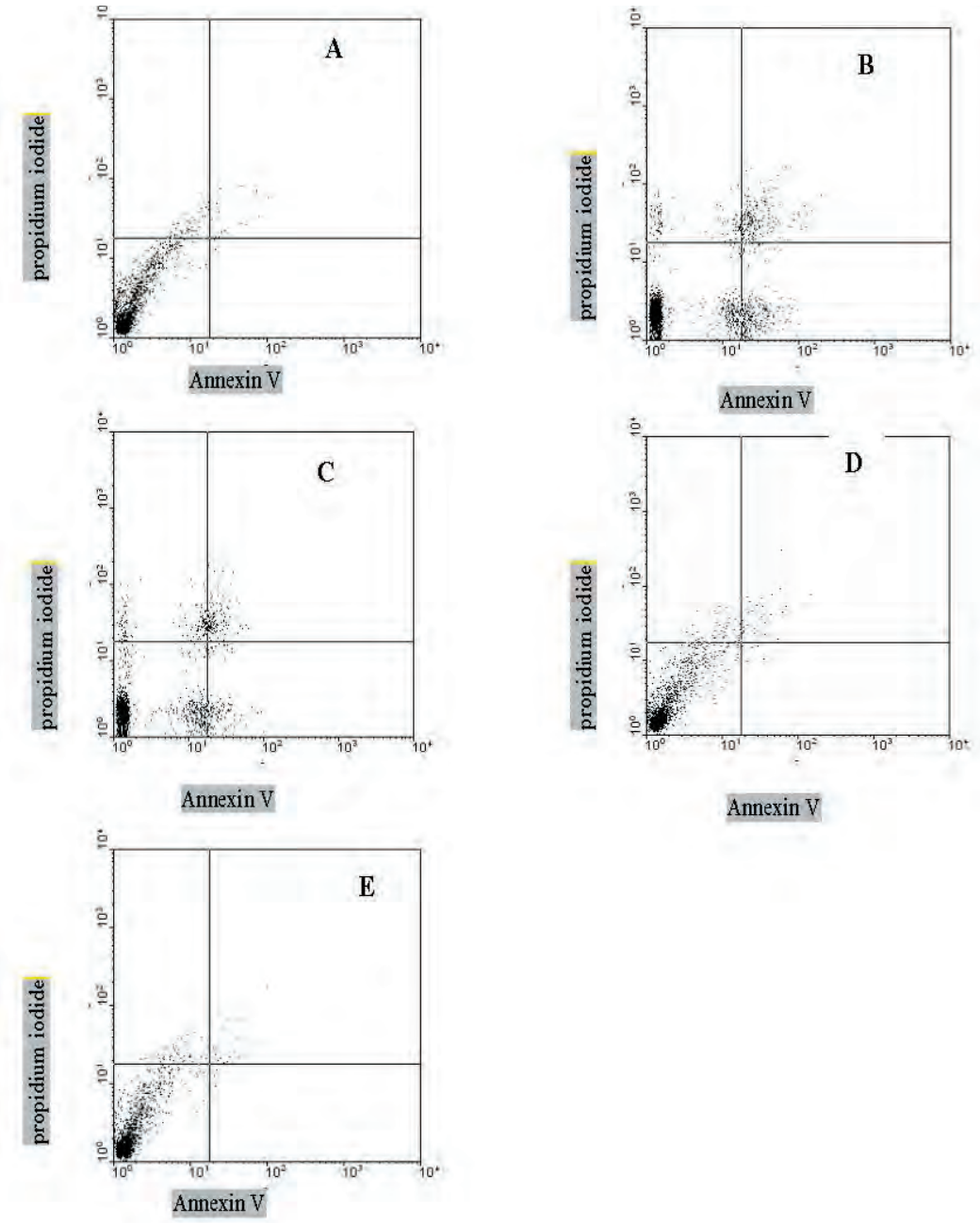


Figure 2: *Apoptosis dot plot*. (A) Control; (B) BPA; (C) BPA + Gb; (D) BPA + EX4; (E) EX4. X axis indicates the cells bind to annexin V and Y axis indicates the cells bind to propidium iodide (PI). The upper left quadrant display necrosis, whereas the left lower quadrant displays the healthy cells. The upper right quadrant revealed dead cells and the lower right quadrant display early stage of apoptosis cells.

damaging effects on GSIS. Further, in current examination insulin secretion showed a decreasing trend at doses of exendin-4 increased >4 nM/L. This chosen concentration of exendin-4 in our study is in agreement with those of the others studies on exendin-4 and its effects on insulin resistance, serum glucose and insulin, lipid profile and antioxidant level [52, 53].

Another important finding was that BPA induced a high percentage of apoptosis in pancreatic islet cells and, it has been said that the amount of healthy cells in the islands has been significantly reduced in BPA group. Also, in this study, the exendin-4 recipient groups showed a very little percentage of apoptosis and the amount of healthy cell was significantly higher. In the study of the effects of bisphenol on pancreatic function, in 2013, Liu et al. demonstrated that functional abnormality occurred in beta cells, could be attributed to BPA impacts on beta

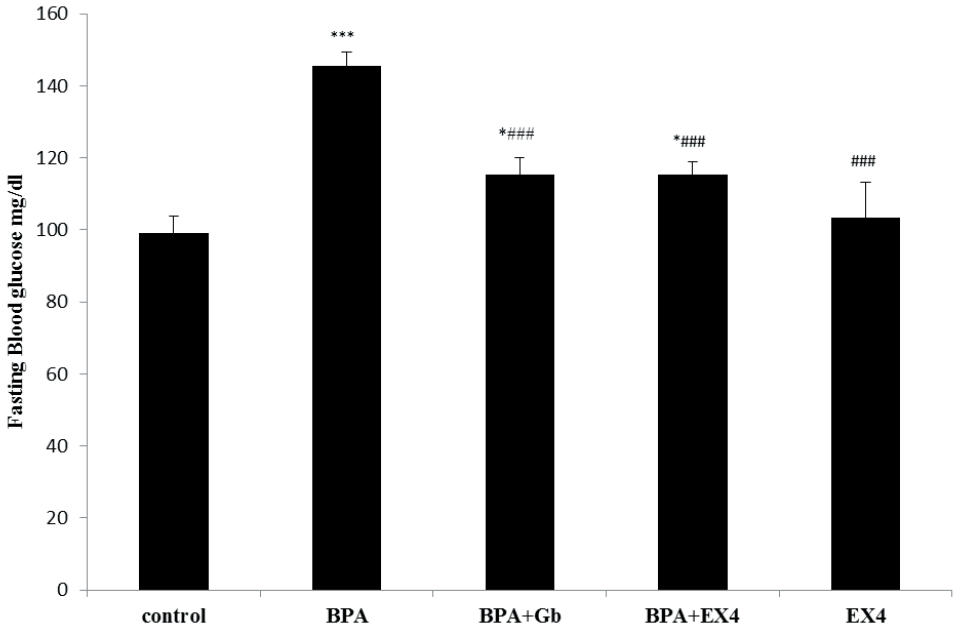


Figure 3: *Effect of BPA and exendin-4, singly and together, on fasting blood glucose in five in vivo groups.* *p<0.05, *** p<0.001 vs. control. ### p<0.001 vs. BPA group. Data is expressed as mean ± SEM.

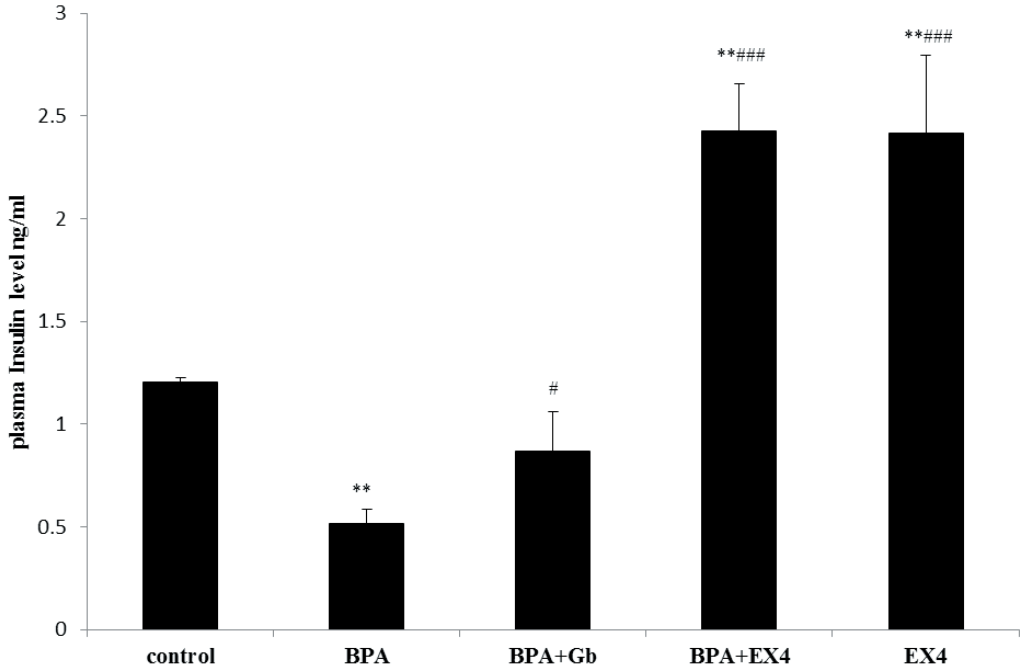


Figure 4: *Effect of BPA and exendin-4, singly and together, on plasma insulin level in five in vivo groups.* ** p<0.01 vs. control. # p<0.05, ### p<0.001 vs. BPA group. Data is expressed as mean ± SEM.

cell apoptosis [54]. Several pathways have been demonstrated as intermediaries for cell apoptosis. Studies by Song et al. have shown that BPA by coupling with ER, induces the stimulatory effect on insulin secretion, and increasing demand for insulin can cause mitochondrial dysfunction in pancreatic beta cells, [46] and over-repetition of these stages resulted in apoptosis of beta cells [55]. Previous studies have reported that exendin-4 by inhibiting mitogen-activated protein kinase kinase 7 and 4 (MKK7 & 4) and reducing G-protein-coupled receptor 40 (GPR40) expression can prevent from apoptosis of beta cells [56]. MKK isoforms are involved in signal transduction

https://doi.org/10.46389/rjd-2020-1048
© 2020 The Authors

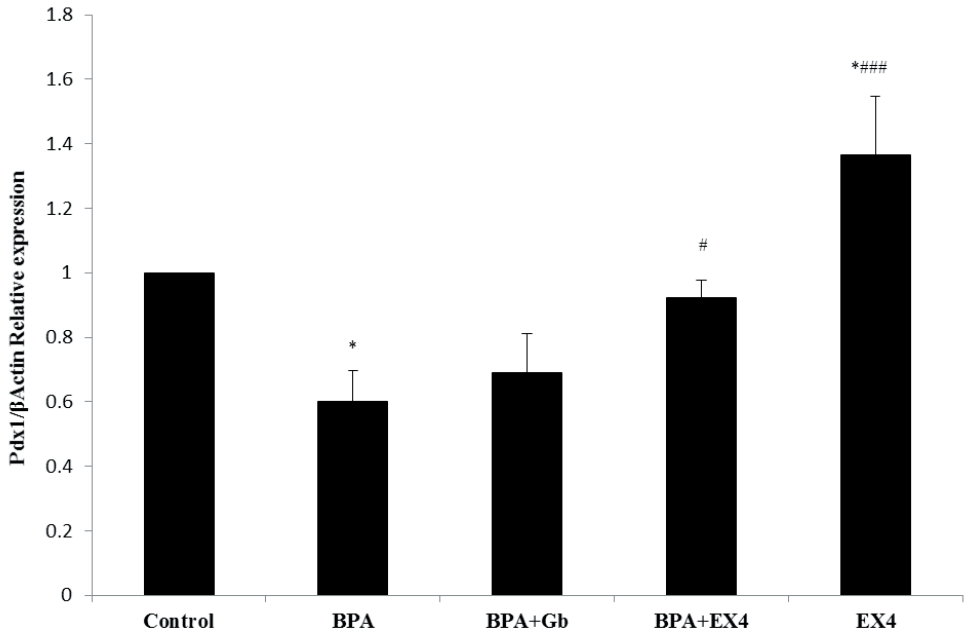


Figure 5: *Effect of BPA and exendin-4, singly and together on Pdx1/βActin relative expression ratio in five in vivo groups.* * p<0.05, vs. control. # p<0.05, ### p<0.001 vs. BPA group. Data is expressed as mean ± SEM.

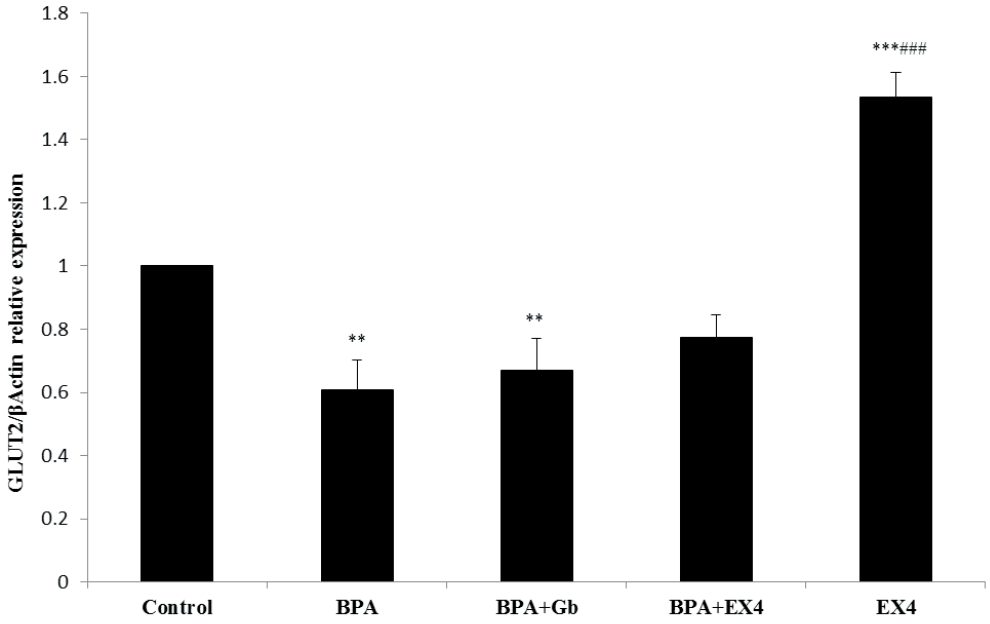


Figure 6: *Effect of BPA and exendin-4, alone and together, on GLUT2/βActin relative expression ratio in five in vivo groups.* ** p<0.01, *** p<0.01 vs. control. ### p<0.001 vs. BPA group. Data is expressed as mean ± SEM.

mediating the cellular responses to proinflammatory cytokines, and environmental stresses [57]. GPR40 plays an important role in obesity and type 2 diabetes, and also in over expression of GPR40 in beta cells leading to diabetes [58]. Carlessi R, et al. in 2015 noted the effects of exendin-4 on improving the health of beta cell by reducing the pancreatic inflammation and oxidative stress, which leads to reduction in ER stress and likelihood of cell death [59].

In the in-vivo conditions, it was observed that BPA had significantly (p<0.001) increased fasting blood glucose levels in the groups receiving it. Several studies investigating that, BPA causes insulin resistance and hyperglycemia by down regulating insulin receptors, glucose

transporter and reducing their function (9, 50, 60). As noted in the results, exendin-4 was able to prevent hyperglycemia induced by BPA, and in this case was equal to glibenclamide. Data from several sources have identified that exendin-4 differentiates pancreatic duct cells into insulin producing cells [61–63]. Conversely, a different study concluded that the glucose lowering effect of exendin-4 in normal, non-diabetic mice appeared did not correlate with increased beta cell mass or insulin secretion [64].

Contrary to what we found, in a different work by Angle et al., BPA caused an increase in plasma insulin level [65] but there were differences in dose and duration of that treatment in our protocol, whereas the findings of D'Cruz et al. were similar to our findings in the current and in our previous experiment [40, 66]. Current knowledge about BPA is equivocal and the duration and dose of exposure are important factors in establishing the type and severity of complications. Further, in the current experiment, alone exendin-4 significantly increased plasma insulin levels. Consistent with our findings, several studies indicated that exendin-4 improved glucose tolerance by elevating plasma insulin levels [67–69].

GLUT2 plays an essential role in GSIS in pancreas β-cells by facilitating the entrance of glucose into the cells [70]. Another study by Moshtagh et al. on adipose-derived tissue stem cells showed that exendin-4 induced expression of Pdx1 and GLUT2 in differentiated cells [71]. In a different work, Chen et al. observed that activation of some cascade pathways in pancreatic β-cells may be important for GLUT2 gene transcription induction by exendin-4, indicating that exendin-4 by activating the CaMKK/CaMKIV cascade (calcium/calmodulin-dependent (CaM) kinase cascade) plays an important role in GK and GLUT2 expression and improvement of insulin secretion in pancreatic β-cells [72]. Recent evidence documented that exendin-4 progress the expression of some important transcription factors, such as Pdx1 and Glut2, suggesting that exendin-4 facilitates differentiation of R1 embryonic stem cells into insulin-producing cells during regeneration [73]. Based on Johnson's studies, the over expression of GLUT2 may be secondary to the up regulation of Pdx1, because Pdx1is a β-cell master gene that has been shown to regulate GLUT2 transcription in β-cells [74, 75]. After increasing of GLUT2 expression in the pancreas and subsequently increasing the glucose transport from beta cells, a signal will be produced, and consequently, insulin secretion will be increased and blood glucose level will be decreased to an extent that glucose homeostasis will be established. Conversely, if it is assumed that GLUT2 also shows over expressed in the intestinal lumen and other tissues, such as kidney tubules; the increase in the absorption and reabsorption of glucose in the blood will also cause more release of insulin from pancreatic beta cells. This can be taken into account in the dramatic increase in insulin levels in groups that received exendin-4.

Conclusions

The purpose of the current study was to determine the effective insulin tropic amount of exendin-4 in the In-vitro and its generalization to the In-vivo, as well as healing effects of exendin-4 on the biochemical and genetic parameters in terms of our existing conditions. These finding suggest that BPA causes increase in the blood glucose level, and also decrease the Pdx1 and GLUT2 gene expression in pancreatic tissue, whereas exendin-4 revealed a preventive role in these cases. This research provides a framework for future study in more potential mechanistic insights of the signaling pathways and more protein and gene evaluation.

Authorship

Authors' contributions: Golshan Afshari and Akram Ahangarpour designed the study, collected the data, conducted the statistical analysis and wrote the manuscript. Golshan Afshari contributed to the statistical analysis, contributed to the discussion, and reviewed the manuscript. Seyyed Ali Mard, Ali Khodadadi, and Mahmoud Hashemitabar contributed to the study design and reviewed the manuscript. All authors read and approved the final version.

https://doi.org/10.46389/rjd-2020-1048
© 2020 The Authors

Conflict of interest

The authors declare no conflict of interest.

Acknowledgment

This study is a part of the Ph.D. thesis of Golshan Afshari and financially supported by vice chancellor of research affairs of the Ahvaz Jundishapur University of Medical Sciences (AJUMS) (Grant No.CMRC-96). The authors also thank the experienced personnel of the Cellular and Molecular Research Center of AJUMS.

References

1. Soltysik K, Czekaj P. Membrane estrogen receptors - is it an alternative way of estrogen action? *J Physiol Pharmacol*. 64(2):129–42, 2013.
2. Duong V, Licznar A, Margueron R, Boulle N, Busson M, Lacroix M, et al. ERalpha and ERbeta expression and transcriptional activity are differentially regulated by HDAC inhibitors. *Oncogene*. 25(12):1799–806, 2006.
3. Kim JH, Cho HT, Kim YJ. The role of estrogen in adipose tissue metabolism: insights into glucose homeostasis regulation. *Endocrine journal*. 61(11):1055–67, 2014.
4. Guillaume M, Montagner A, Fontaine C, Lenfant F, Arnal JF, Gourdy P. Nuclear and Membrane Actions of Estrogen Receptor Alpha: Contribution to the Regulation of Energy and Glucose Homeostasis. *Advances in experimental medicine and biology*. 1043:401–26, 2017.
5. Barros RP, Machado UF, Warner M, Gustafsson JA. Muscle GLUT4 regulation by estrogen receptors ERbeta and ERalpha. Proceedings of the National Academy of Sciences of the United States of America. 103(5):1605–8, 2006.
6. Heine PA, Taylor JA, Iwamoto GA, Lubahn DB, Cooke PS. Increased adipose tissue in male and female estrogen receptor-alpha knockout mice. *Proceedings of the National Academy of Sciences of the United States of America*. 97(23):12729–34, 2000.
7. Diamanti-Kandarakis E, Bourguignon JP, Giudice LC, Hauser R, Prins GS, Soto AM, et al. Endocrine-disrupting chemicals: an Endocrine Society scientific statement. *Endocrine reviews*.30(4):293–342, 2009.
8. Alonso-Magdalena P, Morimoto S, Ripoll C, Fuentes E, Nadal A. The Estrogenic Effect of Bisphenol A Disrupts Pancreatic β-Cell Function In Vivo and Induces Insulin Resistance. Environ Health Perspect. 114(1):106–12, 2005.
9. Alonso-Magdalena P, Morimoto S, Ripoll C, Fuentes E, Nadal A. The estrogenic effect of bisphenol A disrupts pancreatic beta-cell function in vivo and induces insulin resistance. *Environ Health Perspect*. 114(1):106–12, 2006.
10. Alonso-Magdalena P, Ropero AB, Soriano S, Quesada I, Nadal A. Bisphenol-A: a new diabetogenic factor? *Hormones (Athens)*. 9(2):118–26, 2010.
11. Brotons JA, Olea-Serrano MF, Villalobos M, Pedraza V, Olea N. Xenoestrogens released from lacquer coatings in food cans. *Environ Health Perspect*. 103(6):608–12, 1995.
12. Shankar A, Teppala S. Relationship between urinary bisphenol A levels and diabetes mellitus. *The Journal of clinical endocrinology and metabolism*. 96(12):3822–6, 2011.
13. Nadal A, Alonso-Magdalena P, Soriano S, Ripoll C, Fuentes E, Quesada I, et al. Role of estrogen receptors alpha, beta and GPER1/GPR30 in pancreatic beta-cells. *Frontiers in bioscience* (Landmark edition). 16:251–60, 2011.
14. Khan S, Beigh S, Chaudhari BP, Sharma S, Aliul Hasan Abdi S, Ahmad S, et al. Mitochondrial dysfunction induced by Bisphenol A is a factor of its hepatotoxicity in rats. *Environmental toxicology*. 31(12):1922–34, 2016.
15. Lin Y, Sun X, Qiu L, Wei J, Huang Q, Fang C, et al. Exposure to bisphenol A induces dysfunction of insulin secretion and apoptosis through the damage of mitochondria in rat insulinoma (INS-1) cells. *Cell death & disease*. 4:e460, 2013.
16. Lee CY. Glucagon-Like Peptide-1 Formulation--the Present and Future Development in Diabetes Treatment. *Basic & clinical pharmacology & toxicology*. 118(3):173–80, 2016.
17. Parkes DG, Pittner R, Jodka C, Smith P, Young A. Insulinotropic actions of exendin-4 and glucagon-like peptide-1 in vivo and in vitro. *Metabolism: clinical and experimental*. 50(5):583–9, 2001.
18. Goke R, Fehmann HC, Linn T, Schmidt H, Krause M, Eng J, et al. Exendin-4 is a high potency agonist and truncated exendin-(9-39)-amide an antagonist at the glucagon-like peptide 1-(7-36)-amide receptor of insulin-secreting beta-cells. *J Biol Chem*. 268(26):19650–5, 1993.
19. Baggio L, Adatia F, Bock T, Brubaker PL, Drucker DJ. Sustained expression of exendin-4 does not perturb glucose homeostasis, beta-cell mass, or food intake in metallothionein-preproexendin transgenic mice. *J Biol Chem*. 275(44):34471–7, 2000.
20. Wei Q, Sun YQ, Zhang J. Exendin-4, a glucagon-like peptide-1 receptor agonist, inhibits cell apoptosis induced by lipotoxicity in pancreatic beta-cell line. *Peptides*. 37(1):18–24, 2012.
21. Ah Kim H, Lee S, Park JH, Lee S, Lee BW, Ihm SH, et al. Enhanced protection of Ins-1 beta cells from apoptosis under hypoxia by delivery of DNA encoding secretion signal peptide-linked exendin-4. *Journal of drug targeting*. 17(3):242–8, 2009.
22. D'Amour KA, Bang AG, Eliazer S, Kelly OG, Agulnick AD, Smart NG, et al. Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. *Nature biotechnology*. 24(11):1392–401, 2006.
23. Ma J, Chen M, Wang J, Xia HH, Zhu S, Liang Y, et al. Pancreatic duodenal homeobox-1 (PDX1) functions as a tumor suppressor in gastric cancer. *Carcinogenesis*. 29(7):1327–33, 2008.
24. Stolarczyk E, Le Gall M, Even P, Houllier A, Serradas P, Brot-Laroche E, et al. Loss of sugar detection by GLUT2 affects glucose homeostasis in mice. *PloS one*. 2(12):e1288, 2007.
25. Tiano JP, Tate CR, Yang BS, DiMarchi R, Mauvais-Jarvis F. Effect of targeted estrogen delivery using glucagon-like peptide-1 on insulin secretion, insulin sensitivity and glucose homeostasis. *Scientific reports*. 5:10211, 2015.
26. Ahmed-Sorour H, Bailey CJ. Role of ovarian hormones in the long-term control of glucose homeostasis. Interaction

with insulin, glucagon and epinephrine. *Hormone research*. 13(6):396–403, 1980.
27. Lee YS, Shin S, Shigihara T, Hahm E, Liu MJ, Han J, et al. Glucagon-like peptide-1 gene therapy in obese diabetic mice results in long-term cure of diabetes by improving insulin sensitivity and reducing hepatic gluconeogenesis. *Diabetes*. 56(6):1671–9, 2007.
28. Zhu L, Brown WC, Cai Q, Krust A, Chambon P, McGuinness OP, et al. Estrogen treatment after ovariectomy protects against fatty liver and may improve pathway-selective insulin resistance. *Diabetes*. 62(2):424–34, 2013.
29. Carter JD, Dula SB, Corbin KL, Wu R, Nunemaker CS. A practical guide to rodent islet isolation and assessment. *Biol Proced Online*. 11:3–31, 2009.
30. Bodin J, Kocbach Bolling A, Wendt A, Eliasson L, Becher R, Kuper F, et al. Exposure to bisphenol A, but not phthalates, increases spontaneous diabetes type 1 development in NOD mice. *Toxicology reports*. 2:99–110, 2015.
31. Bodin J, Bolling AK, Samuelsen M, Becher R, Lovik M, Nygaard UC. Long-term bisphenol A exposure accelerates insulitis development in diabetes-prone NOD mice. *Immunopharmacology and immunotoxicology*. 35(3):349–58, 2013.
32. Alonso-Magdalena P, Garcia-Arevalo M, Quesada I, Nadal A. Bisphenol-A treatment during pregnancy in mice: a new window of susceptibility for the development of diabetes in mothers later in life. *Endocrinology*. 156(5):1659–70, 2015.
33. Ziv-Gal A, Flaws JA. Evidence for bisphenol A-induced female infertility: a review (2007-2016). *Fertility and sterility*. 106(4):827–56, 2016.
34. Ahangarpour A, Afshari G, Mard SA, Khodadadi A, Hashemitabar M. Alteration Effect of Exendin-4 on Oxidative Stress and Metabolic Disorders Induced by Bisphenol A in Adult Male Mice. *Jentashapir J Health Res*. 7(5):e37836 2016.
35. Adachi T, Yasuda K, Mori C, Yoshinaga M, Aoki N, Tsujimoto G, et al. Promoting insulin secretion in pancreatic islets by means of bisphenol A and nonylphenol via intracellular estrogen receptors. *Food Chem Toxicol*. 43(5):713–9, 2005.
36. Zhang H, Yi Y, Feng D, Wang Y, Qin S. Hypoglycemic Properties of Oxovanadium (IV) Coordination Compounds with Carboxymethyl-Carrageenan and Carboxymethyl-Chitosan in Alloxan-Induced Diabetic Mice. *Evidence-based complementary and alternative medicine: eCAM*.2011:691067, 2011.
37. Li F, Zhang Y, Zhong Z. Antihyperglycemic effect of ganoderma lucidum polysaccharides on streptozotocin-induced diabetic mice. *International journal of molecular sciences*. 12(9):6135–45, 2011.
38. Robertson RP, Harmon J, Tran PO, Tanaka Y, Takahashi H. Glucose toxicity in beta-cells: type 2 diabetes, good radicals gone bad, and the glutathione connection. *Diabetes*. 52(3):581–7, 2003.
39. Taylor JA, Welshons WV, Vom Saal FS. No effect of route of exposure (oral; subcutaneous injection) on plasma bisphenol A throughout 24h after administration in neonatal female mice. *Reproductive toxicology* (Elmsford, NY). 25(2):169–76, 2008.
40. Ahangarpour A, Afshari G, Mard SA, Khodadadi A, Hashemitabar M. Preventive effects of procyanidin A2 on glucose homeostasis, pancreatic and duodenal homebox 1, and glucose transporter 2 gene expression disturbance induced by bisphenol A in male mice. *J Physiol Pharmacol*. 67(2):243–52, 2016.
41. Suzuki R, Tobe K, Terauchi Y, Komeda K, Kubota N, Eto K, et al. Pdx1 expression in Irs2-deficient mouse beta-cells is regulated in a strain-dependent manner. *J. Biol Chem*. 278(44):43691–8, 2003.
42. Rieger AM, Nelson KL, Konowalchuk JD, Barreda DR. Modified annexin V/propidium iodide apoptosis assay for accurate assessment of cell death. *Journal of visualized experiments: JoVE*. (50):2597, 2011.
43. Niu B, Li C, Su H, Li Q, He Q, Liu L, et al. Glucagon-like peptide-1 receptor agonist exendin-4 protects against interleukin-1beta-mediated inhibition of glucose-stimulated insulin secretion by mouse insulinoma beta cells. *Experimental and therapeutic medicine*. 14(3):2671–6, 2017.
44. Padmasekar M, Lingwal N, Samikannu B, Chen C, Sauer H, Linn T. Exendin-4 protects hypoxic islets from oxidative stress and improves islet transplantation outcome. *Endocrinology*. 154(4):1424–33, 2013.
45. Alonso-Magdalena P, Vieira E, Soriano S, Menes L, Burks D, Quesada I, et al. Bisphenol A exposure during pregnancy disrupts glucose homeostasis in mothers and adult male offspring. *Environmental health perspectives*. 118(9):1243–50, 2010.
46. Song L, Xia W, Zhou Z, Li Y, Lin Y, Wei J, et al. Low-level phenolic estrogen pollutants impair islet morphology and beta-cell function in isolated rat islets. *The Journal of endocrinology*. 215(2):303–11, 2012.
47. Casey MF, Neidell M. Disconcordance in statistical models of bisphenol A and chronic disease outcomes in NHANES 2003-08. *PloS one*. 8(11):e79944, 2012.
48. Hassan ZK, Elobeid MA, Virk P, Omer SA, ElAmin M, Daghestani MH, et al. Bisphenol A induces hepatotoxicity through oxidative stress in rat model. Oxid Med Cell Longev. 2012:194829, 2012.
49. Batista TM, Alonso-Magdalena P, Vieira E, Amaral ME, Cederroth CR, Nef S, et al. Short-term treatment with bisphenol-A leads to metabolic abnormalities in adult male mice. *PloS one*. 7(3):e33814, 2012.
50. Alonso-Magdalena P, Ropero AB, Carrera MP, Cederroth CR, Baquie M, Gauthier BR, et al. Pancreatic insulin content regulation by the estrogen receptor ER alpha. *PloS one*. 3(4):e2069, 2008.
51. Alonso-Magdalena P, Quesada I, Nadal A. Endocrine disruptors in the etiology of type 2 diabetes mellitus. *Nature reviews Endocrinology*. 7(6):346–53, 2011.
52. Ahangarpour A, Oroojan AA, Badavi M. Exendin-4 protects mice from D-galactose-induced hepatic and pancreatic dysfunction. *Pathobiology of aging & age related diseases*. 8(1):1418593, 2018.
53. Egido EM, Silvestre RA, Hernandez R, Marco J. Exendin-4 dose-dependently stimulates somatostatin and insulin secretion in perfused rat pancreas. *Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme*. 36(9):595–600, 2004.
54. Liu J, Yu P, Qian W, Li Y, Zhao J, Huan F, et al. Perinatal bisphenol A exposure and adult glucose homeostasis: identifying critical windows of exposure. *PloS one*. 8(5):e64143, 2013.
55. Nadal A, Alonso-Magdalena P, Soriano S, Quesada I, Ropero AB. The pancreatic beta-cell as a target of estrogens and xenoestrogens: Implications for blood glucose homeostasis and diabetes. *Mol Cell Endocrinol*. 304(1–2):63–8, 2009.
56. Natalicchio A, Labarbuta R, Tortosa F, Biondi G, Marrano N, Peschechera A, et al. Exendin-4 protects pancreatic beta cells

 https://doi.org/10.46389/rjd-2020-1048 © 2020 The Authors

from palmitate-induced apoptosis by interfering with GPR40 and the MKK4/7 stress kinase signalling pathway. *Diabetologia*. 56(11):2456–66, 2013.
57. Widenmaier SB, Ao Z, Kim SJ, Warnock G, McIntosh CH. Suppression of p38 MAPK and JNK via Akt-mediated inhibition of apoptosis signal-regulating kinase 1 constitutes a core component of the beta-cell pro-survival effects of glucose-dependent insulinotropic polypeptide. *J Biol Chem*. 284(44):30372–82, 2009.
58. Zhang Y, Xu M, Zhang S, Yan L, Yang C, Lu W, et al. The role of G protein-coupled receptor 40 in lipoapoptosis in mouse beta-cell line NIT-1. *Journal of molecular endocrinology*. 38(6):651–61, 2007.
59. Carlessi R, Lemos NE, Dias AL, Oliveira FS, Brondani LA, Canani LH, et al. Exendin-4 protects rat islets against loss of viability and function induced by brain death. *Molecular and cellular endocrinology*. 412:239–50, 2015.
60. Sakurai K, Kawazuma M, Adachi T, Harigaya T, Saito Y, Hashimoto N, et al. Bisphenol A affects glucose transport in mouse 3T3-F442A adipocytes. *British journal of pharmacology*. 141(2):209–14, 2004.
61. Xu G, Stoffers DA, Habener JF, Bonner-Weir S. Exendin-4 stimulates both beta-cell replication and neogenesis, resulting in increased beta-cell mass and improved glucose tolerance in diabetic rats. *Diabetes*. 48(12):2270–6, 1999.
62. Kim HS, Hong SH, Oh SH, Kim JH, Lee MS, Lee MK. Activin A, exendin-4, and glucose stimulate differentiation of human pancreatic ductal cells. *The Journal of endocrinology*. 217(3):241–52, 2013.
63. Zhou J, Pineyro MA, Wang X, Doyle ME, Egan JM. Exendin-4 differentiation of a human pancreatic duct cell line into endocrine cells: involvement of PDX-1 and HNF3beta transcription factors. *Journal of cellular physiology*. 192(3):304–14, 2002.
64. Fan R, Kang Z, He L, Chan J, Xu G. Exendin-4 improves blood glucose control in both young and aging normal non-diabetic mice, possible contribution of beta cell independent effects. *PloS one*. 6(5):e20443, 2011.
65. Angle BM, Do RP, Ponzi D, Stahlhut RW, Drury BE, Nagel SC, et al. Metabolic disruption in male mice due to fetal exposure to low but not high doses of bisphenol A (BPA): evidence for effects on body weight, food intake, adipocytes, leptin, adiponectin, insulin and glucose regulation. *Reproductive toxicology*. 42:256–68, 2013.
66. D'Cruz SC, Jubendradass R, Jayakanthan M, Rani SJ, Mathur PP. Bisphenol A impairs insulin signaling and glucose homeostasis and decreases steroidogenesis in rat testis: an in vivo and in silico study. Food and chemical toxicology: an international journal published for the British *Industrial Biological Research Association*. 50(3–4):1124–33, 2012.
67. Kwon DY, Kim YS, Ahn IS, Kim da S, Kang S, Hong SM, et al. Exendin-4 potentiates insulinotropic action partly via increasing beta-cell proliferation and neogenesis and decreasing apoptosis in association with the attenuation of endoplasmic reticulum stress in islets of diabetic rats. *Journal of pharmacological sciences*. 111(4):361–71, 2009.
68. Zhou J, Wang X, Pineyro MA, Egan JM. Glucagon-like peptide 1 and exendin-4 convert pancreatic AR42J cells into glucagon- and insulin-producing cells. *Diabetes*. 48(12):2358–66, 1999.
69. Greig NH, Holloway HW, De Ore KA, Jani D, Wang Y, Zhou J, et al. Once daily injection of exendin-4 to diabetic mice achieves long-term beneficial effects on blood glucose concentrations. *Diabetologia*. 42(1):45–50, 1999.
70. Wang X, Zhou J, Doyle ME, Egan JM. Glucagon-like peptide-1 causes pancreatic duodenal homeobox-1 protein translocation from the cytoplasm to the nucleus of pancreatic beta-cells by a cyclic adenosine monophosphate/protein kinase A-dependent mechanism. *Endocrinology*. 142(5):1820–7, 2001.
71. Moshtagh PR, Emami SH, Sharifi AM. Differentiation of human adipose-derived mesenchymal stem cell into insulin-producing cells: an in vitro study. *Journal of physiology and biochemistry*. 69(3):451–8, 2013.
72. Chen K, Yu X, Murao K, Imachi H, Li J, Muraoka T, et al. Exendin-4 regulates GLUT2 expression via the CaMKK/CaMKIV pathway in a pancreatic beta-cell line. *Metabolism: clinical and experimental*. 60(4):579–85, 2011.
73. Zhao Q, Yang Y, Hu J, Shan Z, Wu Y, Lei L. Exendin-4 enhances expression of Neurod1 and Glut2 in insulin-producing cells derived from mouse embryonic stem cells. *Archives of medical science: AMS*. 12(1):199–207, 2016.
74. Johnson JD, Ahmed NT, Luciani DS, Han Z, Tran H, Fujita J, et al. Increased islet apoptosis in Pdx1+/- mice. *The Journal of clinical investigation*. 111(8):1147–60, 2003.
75. Johnson JD et al. Insulin protects islets from apoptosis via Pdx1 and specific changes in the human islet proteome. *Proceedings of the National Academy of Sciences of the United States of America*. 103(51):19575–80, 2006.

© 2020 The Authors

https://doi.org/10.46389/rjd-2020-1049
www.rjdnmd.org

Rom J Diabetes Nutr Metab Dis
2020; volume 27, issue 4, pages 336-341

Original Research

Glucose dependent insulinotropic polypeptide in impaired glucose tolerance and its association with insulin secretion and sensitivity

Marufa Akhter[1], Zebunnesa Zeba[2], Mamun Mia[3], Salima Akter[3,5]*, Rahelee Zinnat[4], Liaquat Ali[4]

[1] Department of Biochemistry, Ad-din Sakina Women's Medical College, Jashore 7400, Bangladesh
[2] Department of Public Health and Informatics, Jahangirnagar University, Savar 1342, Bangladesh
[3] Department of Medical Biotechnology, Bangladesh University of Health Sciences, Dhaka 1216, Bangladesh
[4] Department of Biochemistry and Cell Biology, Bangladesh University of Health Sciences, Dhaka 1216, Bangladesh
[5] Department of Biochemistry and Molecular Biology, School of Medicine, Kyung Hee University, Seoul, Republic of Korea

*Correspondence to: Salima Akter, Department of Medical Biotechnology, Bangladesh University of Health Sciences (BUHS). 125/1-Darus Salam Rd, Dhaka 1216, Bangladesh. Phone: (880)2-9010932, 9010952. Fax: [880]2-8055312 and Department of Biochemistry and Molecular Biology, School of Medicine, Kyung Hee University, 26 Kyungheedae-ro, Dongdaemun-gu, Seoul 02447, Republic of Korea. Phone: 82-2-961-0524. Fax: 82-2-959-8168. E-mail: salima_2015@buhs.ac.bd

Received: 17 April 2020 / Accepted: 21 July 2020

Abstract

Background and Aims: The abnormalities of incretin effects have been established as major determinants of insulin secretion and sensitivity which starts early in prediabetes. However, the pathophysiology of these states with incretin in Bangladeshi population has only been started to be investigated. The present study was undertaken to explore the association of glucose dependent insulinotropic polypeptide (GIP) with glycemic and insulinemic status in impaired glucose tolerance (IGT). **Material and Methods:** The analytic observational study was conducted under a case-control design with age and body mass index (BMI) matched 51 IGT and 47 control subjects. Serum C-peptide and GIP were measured by enzyme linked immunosorbent assay (ELISA). Insulin secretory capacity (HOMA-%B) and insulin sensitivity (HOMA-%S) were calculated by homeostasis model assessment. **Results:** IGT subjects showed significantly higher serum fasting GIP (FGIP) level compared to controls [pgm/ml, 74.2 (10.0–190.0) vs. 49.6 (6.1–278.0), (p= 0.001)]. There was no significant difference of post glucose GIP (PGIP) in the study subjects, however, the ratio analysis revealed reduced secretion of PGIP to FGIP (p<0.001), and fasting C-peptide (FC-peptide) to FGIP (p< 0.05), respectively in IGT. In addition, serum PGIP and 2 h postload serum glucose (2 h PG) ratio was also significantly reduced in IGT compared to controls [7.00 (2.87–18.42) vs. 10.08 (1.88–23.25), (p<0.01)]. In multiple regression, a significant positive association between FC-peptide and FGIP (p<0.01) and negative association between FGIP and HOMA-%S (p= 0.05) were demonstrated. **Conclusion:** The incretin effect of GIP is diminished in IGT and it is associated with insulin resistance in Bangladeshi type 2 diabetic population.

Keywords: Glucose dependent insulinotropic polypeptide (GIP), Insulin secretion, IGT, Insulin sensitivity.

Background and Aims

Diabetes mellitus (DM) is a major health burden all over the world increasing to epidemic levels particularly in the developing countries [1]. The pathophysiology as well as risk factors of the disorder has shown considerable heterogeneity depending on racial, environmental, demographic, socioeconomic, and cultural factors [2]. GIP, one of the primary incretin hormone secreted from the intestine [3, 4], stimulates insulin secretion in a glucose-dependent manner [5–7]. Approximately 70% of the overall post-prandial insulin response to glucose is mediated by GIP with the help of glucagon-like peptide-1 (GLP-1), another

© 2020 The Authors. Romanian Journal of Diabetes, Nutrition and Metabolic Diseases published by Sanatatea Press Group on behalf of the Romanian Society of Diabetes Nutrition and Metabolic Diseases. This is an open access article under the terms of the Creative Commons Attribution License (CC-BY-NC-ND 3.0).

member of the incretion hormone family [8]. Moreover, GIP stimulates proinsulin gene transcription and translation [9, 10], and act as a β-cell mitogenic and anti-apoptotic factor [11]. Incretin defects have been found to be associated with type 2 diabetes mellitus (T2DM) from its early stage [12]; however, the causal relationship between incretin and T2DM has still remain controversial as the studies have shown variable results [12–15]. One of the approaches to resolve the issue is to investigate the incretin hormone profile in subjects with impaired fasting glucose (IFG) and (IGT) who are considered to have a prediabetes status with a progression rate of 5 to 10 percent to DM every annum [16].

Bangladesh now ranks 10th in the total number of diabetic population with vast majority being of T2DM and the number is increasing very rapidly [1]. Studies on prediabetic subjects have shown that both the basic defects of T2DM, i.e. insulin secretory abnormality and insulin resistance, are present in Bangladeshi IFG and IGT subjects in variable degrees [17] with the secretory defect being highly predominant in the former group [18]. The association of incretin hormones with insulin secretion or insulin sensitivity has so far not been investigated in any of the prediabetic groups. Thus, the present study was undertaken to investigate whether basal and post stimulatory GIP profile has any relation with glycemic and insulinemic status.

Materials and Methods

Study design and subjects

An observational study with a case-control design was conducted in the biomedical research group, department of Biochemistry & Cell Biology, Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders (BIRDEM), Dhaka, Bangladesh. Voluntarily agreed adult subjects with age ranging from 30 to 55 years, were included after taking informed consent and a total number of 51 IGT subjects and 47 control subjects were recruited in the study. The two groups were matched for age and BMI. Diabetes and prediabetes were diagnosed following WHO Group Study criteria [19]. Subjects with serious co-morbid diseases like severe infection, stroke, myocardial infarction, major surgery, malabsorption, history of using drugs significantly affecting glucose metabolism (glucocorticoids, oral contraceptives containing levonorgestrel or high dose estrogen, phenytoin and high dose thiazide diuretics etc.) and pregnant women were excluded. All subjects underwent standard procedures of anthropometric measurements like body weight, height, waist and hip circumference (WC and HC).

Biochemical analysis

After overnight fasting (8–14 h), blood samples were collected by venipuncture to assess the biochemical tests including fasting and 2 h post-load (75 g glucose) glucose. All tests were measured by standard laboratory methods using a conventional automated analyzer (Dimension XL® clinical chemistry system, Siemens Healthcare Diagnostics Inc. USA). Serum C-peptide and serum GIP were measured by ELISA technique using commercial kits (DRG-International, Germany). For beta cell assessment, insulin secretory capacity (HOMA-%B) and insulin sensitivity (HOMA-%S) were estimated by homeostasis model assessment using HOMA-SIGMA software.

Statistical Analysis

Data were expressed as mean ± standard deviation (SD) and/or median (range) wherever appropriate. Comparison of mean values between two groups was tested using either Student's 't' test (Unpaired) or Mann-Whitney 'U' test. Bivariate correlation analysis was done by using Spearman's correlation analysis. Univariate regression analysis was performed taking C-peptide and HOMA-%S as dependent variable and others as independent/confounding variables as appropriate. All statistical measures were performed using statistical package for social science (SPSS) for windows version 11.5.

© 2020 The Authors

Table 1: Anthropometric and clinical characteristics of the study subjects.

Variables	Control (n = 47)	IGT (n = 51)	z/p value
Age (yrs)	40±6	41±5	0.673/0.502
BMI (kg/m^2)	24.0±2.9	24.0±3.5	0.570/0.955
WHR	0.88±0.05	0.92±0.08	2.340/0.02
FSG (mmol/l)	5.2 (4.1–6.0)	5.3 (4.4–6.0)	0.502/0.615
2 h PG (mmol/l)	6.3 (4.8–7.8)	9.3 (7.9–11.0)	8.502/0.001
FC-pep (pmol/l)	0.64 (0.18–1.62)	0.68 (0.21–1.39)	1.000/0.310
HOMA-%B	117.0 (58.4–461.0)	117.0 (39.4–455.0)	0.109/0.914
HOMA-%S	71.0 (27.0–247.0)	65.0 (22.0–222.0)	1.890/0.050
FGIP (pgm/ml)	49.6 (6.1–278.0)	74.2 (10.0–189.9)	3.30/0.001
PGIP (pgm/ml)	267.0 (37.8–700.4)	255.0 (131.0–616.3)	0.175/0.861
z/p value of FGIP and PGIP	5.958/0.001	6.205/0.001	

Results were expressed as mean ± SD or median (range). Unpaired Student's 't' test and Mann –Whitney U test were performed to compare between groups and the test of significance at 5% significance level. n, number of subjects; IGT, impaired glucose tolerance; BMI, body mass index; WHR, waist hip ratio; FSG, fasting serum glucose; 2 h PG, 2 hour post-load (75 g) glucose; serum glucose; FC-pep, fasting C-peptide; HOMA-%B, β cell secretory capacity; HOMA-%S, insulin sensitivity by homeostasis model assessment; FGIP, fasting serum glucose dependent insulinotropic polypeptide; PGIP, post glucose serum glucose dependent insulinotropic polypeptide.

Results

Characteristics of the study subjects

The clinical characteristics of the controls and subjects with isolated IGT are shown in Table 1. Two groups were matched for age and BMI (p = 0.955). The IGT group showed significantly higher WHR as compared to the control group [(0.92±0.08 vs. 0.88±0.05), p = 0.02]. IGT subjects had reduced HOMA-%S index compared to controls [65.0 (22.0–222.0) vs. 71.0 (27.0–247.0), p = 0.050], but HOMA-%B was not significantly different between the groups (Table 1). Fasting GIP was significantly higher in subjects with IGT compared to controls [pgm/ml; 74.2 (10.0–189.9) vs. 49.6 (6.1–278.0), p = 0.001]. In contrast, subjects with normal glucose tolerance and IGT did not show any significant difference in post glucose GIP levels (p = 0.861) (Table 1).

C-peptide, glucose and GIP ratios of the study subjects

Subjects with IGT exhibited significantly reduced serum fasting C-peptide and fasting GIP ratio than the control subjects [0.037 (0.009–0.251) vs. 0.045 (0.009–0.205), p = 0.050)] but fasting C-peptide-glucose ratio was comparable (Table 2). The fasting GIP/glucose ratio was significantly higher in IGT [3.53 (0.41–7.96) vs. 2.26 (0.29–11.26), p=0.002]; however, postglucose GIP/glucose was significantly lower in IGT compared to controls [7.0 (2.87–18.42) vs. 10.08 (1.88–23.25), p<0.01]. Similarly, the ratio of post glucose GIP with fasting GIP was significantly lower in IGT compared to controls [3.47 (0.98–22.0) vs. 5.14 (0.96–19.85), p < 0.01] (Table 2).

Multiple regression analysis of the association of C-peptide and HOMA-%S with variables of interest of the study subjects:

On regression analysis, a significant positive association was found between fasting C-peptide and fasting GIP (p < 0.01) (Table 3).

A significant negative association was found between fasting GIP and insulin sensitivity (HOMA-%S) (p = 0.05) (Table 4).

No significant association was found between fasting glucose and fasting GIP in both

https://doi.org/10.46389/rjd-2020-1049
© 2020 The Authors

Table 2: C-peptide, Glucose and GIP ratios of the study subjects.

Variables	Control (n = 47)	IGT (n = 51)	z/p value
FC-pep : FSG	0.120 (0.04–0.30)	0.123 (0.03–0.29)	0.639/0.523
FC-pep : FGIP	0.045 (0.009–0.205)	0.037 (0.009–0.251)	1.95/0.050
FGIP : FSG	2.26 (0.29–11.26)	3.53 (0.41–7.96)	3.07/0.002
PGIP : PSG	10.08 (1.88–23.25)	7.00 (2.87–18.42)	4.46/0.001
PGIP : FGIP	5.14 (0.96–19.85)	3.47 (0.98–22.0)	4.469/0.001

Results were expressed as median (range). Mann-Whitney U test was performed and the test of significance at 5% significance level. n number of subjects; IGT, impaired glucose tolerance; FC-pep, fasting C-peptide; FSG, fasting serum glucose. FGIP, fasting glucose dependent insulinotropic polypeptide; 2 h PG, 2-hour post-load serum glucose; PGIP, post glucose serum glucose dependent insulinotropic polypeptide.

Table 3: Multiple regression analysis of the association of fasting C-peptide with variables of interest of the study subjects.

Variables	Model 1		Model 2		Model 3		Model 4	
	β	p	β	p	β	p	β	p
Intercept		0.001		0.215		0.767		0.882
FGIP (pgm/ml)	0.343	0.002	0.307	0.006	0.306	0.006	0.298	0.009
FSG (mmol/l)			0.177	0.106	0.147	0.189	0.136	0.242
WHR					0.124	0.258	0.113	0.327
Group							–0.042	0.732
Adjusted R^2	**0.106**		**0.125**		**0.129**		**0.118**	

Standardized regression coefficients (β) were given with the level of significance. R^2 for adjusted R square (Multiple coefficient of determination). FSG, fasting serum glucose; FGIP, fasting GIP; WHR, waist to hip ratio.

Table 4: Multiple regression analysis of the association of HOMA-%S with variables of interest of the study subjects.

Variables	Model 1		Model 2		Model 3	
	β	p	β	p	β	p
Intercept		0.001		0.109		0.153
FGIP (ng/ml)	–0.253	0.024	–0.246	0.028	–0.218	0.050
WHR			–0.106	0.338	–0.066	0.573
Group					0.121	0.319
Adjusted R^2	**0.052**		**0.051**		**0.051**	

The level of significance at $p<0.05$.

simple correlation and multiple regression (data not shown).

Discussion

This study investigated the association of GIP with insulin secretion and sensitivity in Bangladeshi IGT subjects. The present study reveals that GIP secretion is up regulated in the fasting state. This is evident both in terms of absolute GIP and C-peptide to GIP ratio values at the fasting state when compared between control and IGT groups (Table 2). The importance of GIP has mostly been conceived in relation to nutrient intake [7, 20–25], but its role in the maintenance of glucose homeostasis in the fasting state has been discussed less. In the present study,

© 2020 The Authors

fasting serum glucose level does not differentiate between control and IGT groups with a minimal rise in fasting C-peptide levels in the later group. The secretory capacity of the pancreatic β cells is not significantly different between the two groups. However, data showed that insulin sensitivity, as assessed by HOMA-%S, is compromised in IGT subjects. Under these conditions it will be important to explore whether raise of serum GIP plays any role in preventing the rise of fasting serum glucose in the IGT group.

Our data is comparable with a previous study [26] that revealed hypersecretion of GIP at fasting state but normal secretion of plasma insulin in IGT subjects. Nonetheless, after 40 minutes of OGTT, plasma insulin level was increased with a later rise of C-peptide value. But they have reported normal plasma insulin concomitant with decreased GIP level in T2DM. The pathophysiology lies here that long term insulin receptor resistance leads to defective signaling of the glucose sensing genes both in β-cells and GIP secretory K cells of the duodenum resulting in β-cell failure and dysregulation of GIP, respectively.

Although the present work has demonstrated the significantly higher FGIP value in IGT compared to controls and both the groups showed acute rise in the GIP values in response to oral glucose load, the difference between two groups were totally lost at the postprandial state. There was a proportionately higher rise of GIP values (about 5 times) in the controls compared to a blunted response (about 3.5 times) in IGT subjects at the corresponding state (Table 2). The abnormality is also evident on ratio analysis where post glucose GIP to 2 h post-load serum glucose (PGIP : 2 h PG) is significantly lower ($p < 0.001$) in the IGT group compared to controls.

In support of the finding in univariate analysis, multiple regression analysis reveals a much stronger association of FC-pep with FGIP ($p<0.001$) as compared to weaker association with HOMA-%S on adjusting the confounding variables of fasting glucose, waist-hip ratio (WHR) and presence of diabetes. Accordingly, it seems that FGIP has much more relevance to the insulin secretory pathway, as reported by some authors [14–15, 26], compared to the insulin action pathway. Nevertheless, further studies are required to clarify these issues.

Analysis of the anthropometric data in the present study shows that the IGT subjects do not have generalized obesity as evident by no difference in BMI between control and IGT groups. IGT, however, is associated with central obesity ($p=0.02$). The finding is comparable with the observations in a previous study conducted on the same as well as in other population [18, 27]. Central obesity is known to be more specifically related to the increased secretion of adipocytokines [28–30] (like resistin and adiponectin) and inflammatory markers (like hs-CRP) which, in turn, are associated with insulin resistance. Thus, consistent finding of central obesity in the IGT population can be a central issue in designing preventing campaigns for reducing abdominal fat through lifestyle and dietary modifications.

The limitations of the study were lack of various groups of impaired glucose regulation (such as IFG and combined IFG & IGT). Postglucose-load serum C-peptide was not analyzed, and glucagon like peptide-1(GLP-1) and GIP were not studied together to estimate their relative contribution in insulin secretion and sensitivity.

Conclusion

In conclusion, the IGT subjects have insulin resistance but their pancreatic B cell function seems to be still uncompromised. GIP secretion in IGT is up regulated at the fasting state and it has a blunted response to oral glucose in this disorder. GIP does not have any association with insulin secretion in IGT, but it has an association with insulin resistance.

Acknowledgments

The authors thank all the participants and laboratory team members for their excellent cooperation and helping attitude for conduction of the study. This work was financially supported by the International Program in the Chemical Sciences (IPICS), Uppsala University, Sweden; Bangladesh Diabetic Somity (BADAS), and the National Research Foundation of Korea (NRF)

 https://doi.org/10.46389/rjd-2020-1049 © 2020 The Authors

grant funded by the Korean government (MEST) (NRF-2020H1D3A1A04080389 to Salima Akter).

Conflict of interests

The authors declare no conflict of interest.

References

1. IDF Diabetes Atlas. Eighth edition: *International Diabetes Federation*, 2017.
2. Roglic G.WHO Global report on diabetes: A summary. *Int J Non-Commun Dis*. 1: 3–8, 2016.
3. Drucker DJ, Nauck MA et al. The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. *Lancet*. 368: 1696–1705, 2006.
4. McIntosh CHS, Widenmaier S, Kim S-J et al. Glucose- dependent insulinotropic polypeptide (gastric inhibitory polypeptide; GIP). *Vitam Horm*. 80: 409–471, 2009.
5. Kolic J, Spigelman AF, Smith AM et al. Insulin Secretion Induced by Glucose-dependent Insulinotropic Polypeptide Requires PI3 Kinase-γ in Rodent and Human β-Cells. *J Biol Chem*. 289: 32109–32120, 2014.
6. O'Harte FP, Abdel- WahabYH, Conlon JM et al. Amino terminal glycation of gastric inhibitory polypeptide enhance its insulinotropic action on clonal pancreatic B-cells. *Biochim et Biophys Acta*. 1425: 319–327, 1998.
7. Nauck MA, Homberger E, Siegel EG et al. Incretin effects of increasing glucose loads in man calculated from venous insulin and C-peptide responses. *J Clin Endocrinol Metab*. 63: 492–498, 1986.
8. Calanna S, Christensen M, Holst JJ et al. Secretion of glucose-dependent insulinotropic polypeptide in patients with type 2 diabetes: systematic review and meta-analysis of clinical studies. *Diabetes Care*. 36: 3346–3352, 2013.
9. Fehmann H-C, Göke B et al. Characterization of GIP (1–30) and GIP (1–42) as stimulators of proinsulin gene transcription. *Peptides*. 16: 1149–1152, 1995.
10. Wang Y, Montrose-Rafizadeh C, Adams L et al. GIP regulates glucose transporters, hexokinases, and glucose-induced insulin secretion in RIN 1046-38 cells. *Mol Cell Endocrinol* 116: 81–87, 1996.
11. Trumper A, Trumper K, Horsch D et al. Mechanisms of mitogenic and anti-apoptotic signaling by glucose-dependent insulinotropic polypeptide in beta (INS-1)-cells. *J Endocrinol*. 174: 233–246, 2002.
12. Michaliszyn SF, Mari A, Lee S et al. β-cell function, incretin effect, and incretin hormones in obese youth along the span of glucose tolerance from normal to prediabetes to type 2 diabetes. *Diabetes*. 63: 3846–3855, 2014.
13. Knop FK, Vilsbøll T, Højberg PV et al. Reduced incretin effect in type 2 diabetes: cause or consequence of the diabetic state? *Diabetes*. 56: 1951–1959, 2007.
14. Meier JJ, Nauck MA et al. Is the diminished incretin effect in type 2 diabetes just an epi-phenomenon of impaired β-cell function? *Diabetes*. 59: 1117–1125, 2010.
15. Rask E, Olsson T, Söderberg S et al. Insulin secretion and incretin hormones after oral glucose in non-obese subjects with impaired glucose tolerance. *Metabolism*. 53: 624–631, 2004.
16. Tabák AG, Herder C, Rathmann W et al. Prediabetes: a high-risk state for diabetes development. *Lancet*. 379: 2279–2290, 2012.
17. Shefin S, Faruque M, Hafizur R et al. Insulin secretory capacity and insulin sensitivity in the normoglycemic first degree relatives of Bangladeshi subjects with prediabetes. *J Diabetol*. 4: 5, 2013.
18. Rahman MH, Hafizur RM, Nahar Q et al. Insulin secretion and sensitivity in Bangladeshi prediabetic subjects. *J Diabetes Complicat*. 24: 37–42, 2010.
19. World Health Organization and International Diabetes Federation. Definition and diagnosis of Diabetes Mellitus and Intermediate Hyperglycemia: WHO/IDF Consultation. Geneva, 2006.
20. Seino Y, Fukushima M, Yabe D et al. GIP and GLP-1, the two incretin hormones: similarities and differences. *J Diabetes Investig*. 1: 08–23, 2010.
21. Cho YM, Kieffer TJ et al. K-cells and glucose-dependent insulinotropic polypeptide in health and disease. *Vitam Horm*, 84: 111–150, 2010.
22. Yabe D, Kuroe A, Watanabe K et al. Early phase glucagon and insulin secretory abnormalities, but not incretin secretion, are similarly responsible for hyperglycemia after ingestion of nutrients. *J diabetes Complicat*. 29: 413–421, 2015.
23. Meier JJ, Hücking K, Holst JJ et al. Reduced insulinotropic effect of gastric inhibitory polypeptide in first-degree relatives of patients with type 2 diabetes. *Diabetes*. 50: 2497–2504, 2001.
24. Ahrén B, Larsson H, Holst JJ et al. Reduced gastric inhibitory polypeptide but normal glucagon-like peptide 1 response to oral glucose in postmenopausal women with impaired glucose tolerance. *Eur J Endocrinol*. 137: 127–131, 1997.
25. Faerch K, Vaag A, Holst JJ et al. Impaired fasting glycaemia vs impaired glucose tolerance: similar impairment of pancreatic alpha and beta cell function but differential roles of incretin hormones and insulin action. *Diabetologia*. 51: 853–861, 2008.
26. Theodorakis MJ, Carlson O, Muller DC et al. Elevated plasma glucose-dependent insulinotropic polypeptide associates with hyperinsulinemia in impaired glucose tolerance. *Diabetes Care*. 27: 1692–1698, 2004.
27. Kleber M, de Sousa G, Papcke S et al. Risk factors for impaired glucose tolerance in obese children and adolescents. *World J Diabetes*. 1: 129–134, 2010.
28. Ouchi N, Ohashi K, Shibata R et al. Adipocytokines and obesity-linked disorders. *Nagoya j Med Sci*. 74: 19–30, 2012.
29. Jung U, Choi M-S et al. Obesity and its metabolic complications: the role of adipokines and the relationship between obesity, inflammation, insulin resistance, dyslipidemia and nonalcoholic fatty liver disease. *Int J Mol Sci*. 15: 6184–6223, 2014.
30. Cao H. Adipocytokines in obesity and metabolic disease. *J Endocrinol*. 220: T47–T59, 2014.

© 2020 The Authors

https://doi.org/10.46389/rjd-2020-1050
www.rjdnmd.org

Rom J Diabetes Nutr Metab Dis
2020; volume 27, issue 4, pages 342-348

Original Research

Study to evaluate the correlation between coagulation factor, glycemic control and the severity of diabetic foot ulcers among South Indian population: A case control study

Santhini Gopalakrishnan[1], Anuradha G[1], Sumathy S[1], Sandeep Unnikrishnan[2]* Dhanvarshini S[3]

[1] **Department Biochemistry, Chettinad Academy of Research and Education, Chennai, India**
[2] **Department Plastic surgery, Chettinad Super Specialty Hospital, Chennai, India**
[3] **Ms Dhanvarshini, Chettinad Academy of Research and Education, Chennai, India**

*Correspondence to: Sandeep Unnikrishnan, Department of Plastic Surgery, Chettinad Super Specialty Hospital, Kelambakkam, Chengalpattu District, Tamil Nadu - 603103, India. E-mail: santhini.dept@gmail.com, Phone: 8754484353

Received: 3 August 2020 / Accepted: 17 October 2020

Abstract

Background: Diabetes mellitus is the most common metabolic disorder characterized by metabolic abnormalities and long term complications. Diabetic foot ulcer(DFU) is an important complication of diabetes mellitus. It contributes to major source of morbidity and mortality among chronic diabetic patients. **Aim:** To find out the utility of fibrinogen, Prothrombin time (PT) and Activated Partial Thromboplastin Time(APTT) as diagnostic markers to assess the severity of Diabetic foot ulcer. **Materials and Methods:** 60 subjects admitted with diabetic ulcer of foot 60 control subjects with diabetes mellitus but without DFU were included in the study. Fibrinogen, Prothrombin time(PT) and Activated Partial thromboplastin time(APTT) were measured in the samples collected from the subjects. **Results:** The mean level of fibrinogen among cases was 452.3±136.4 and among cases was 306.5=/-79.6 respectively and there was statistically significant difference between the two groups. (p value=0.0001). Prothrombin time (PT) showed mean value of 16.68±4.2 sec among cases and 12.5±1.6 sec among controls and the difference was statistically significant (p value=0.0001). APTT showed mean value of 36.2±7.07 sec among cases compared to 34±7.3sec among controls but the difference was not statistically significant (p value=0.24). ROC analysis shows that the area under the curve for HbA1c fibrinogen is 0.758 and 0.760 respectively. **Conclusion:** Fibrinogen and prothrombin time is found to have a significant difference between DFU patients and control subjects in the diagnosis of diabetic foot ulcer.

Keywords: Diabetic Foot Ulcer, Fibrinogen, Prothrombin time, Activated Partial Thromboplastin time.

Background and Aims

Diabetes mellitus is the most common endocrine disorder characterized by metabolic abnormalities and long-term complications. The Diabetes Federation of India has estimated that the number of diabetes patients in India will be about 550 million by 2030 [1, 2].

This disease is associated with many microvascular and macrovascular complications. Eighty percent of deaths among diabetic patients are contributed by thrombotic events, while cardiac problems contribute up to 75%, with the remaining 25% caused due to peripheral vascular events and cerebrovascular complications [9].

Patients with diabetes have a 25% chance of developing diabetic foot ulcers in the future [3]. It is a complication with a mortality rate of approximately 16%, an amputation rate of 25%, and a 3-year mortality rate after amputation is 37% [4].

© 2020 The Authors. Romanian Journal of Diabetes, Nutrition and Metabolic Diseases published by Sanatatea Press Group on behalf of the Romanian Society of Diabetes Nutrition and Metabolic Diseases. This is an open access article under the terms of the Creative Commons Attribution License (CC-BY-NC-ND 3.0).

Diabetic Foot Ulcers (DFU) is a major complication in chronic diabetics with severe consequences [5]. Diabetic foot ulcers development occurs due to the simultaneous effects of many risk factors among which peripheral neuropathy and peripheral arterial disease play a major role [6, 7, 8]. Peripheral arterial disease affects the tibial and peroneal arteries in the calf region. Endothelial dysfunction and smooth muscle cell abnormalities develop in the peripheral arteries due to the uncontrolled hyperglycemic status, which contributes to the development of DFU. Diabetes is a pro-coagulant state where there is an increase in the levels of pro-coagulants like fibrinogen and other clotting factors such as I, VII, IX, XII and Von will brand factor [9]. So, in recent years, the role of hemostatic factors in the development of microvascular and macrovascular complications in diabetes has achieved great interest. Plasma fibrinogen is an important component of the coagulation cascade as well as a major determinant of blood viscosity and blood flow, which in turn is a major determinant of the progress of diabetic foot [10]. Fibrinogen is an inflammatory marker linked to the development of atherosclerosis, thrombosis, and vascular complications in Diabetes mellitus. The complications caused by increased fibrinogen levels are cardiovascular disease, cerebrovascular accidents, and peripheral vascular diseases [11].

Prothrombin time and Activated partial thromboplastin time are two tests used to assess the extrinsic and intrinsic pathways of coagulation. They are analyzed to find out bleeding or clotting tendencies. Studies have shown that levels of these markers are raised among diabetic patients when compared to normal individuals. The diagnosis of diabetic foot ulcers is mainly based on clinical and radiological methods. Although the literature states variations in coagulation profile in Diabetic foot ulcers, there has not been much research on them in this part of the country.

This study aims to find out the levels of fibrinogen, PT, and APTT among DFU patients, and to find out whether there is any correlation between plasma fibrinogen levels with the levels of HbA1c in diabetic foot ulcers subjects among South Indian population.

Material and Method

Ethical approval of the study protocol

Prior to commencing the study, the approval of the Ethical Committee of the Institute was obtained. It was a case control study conducted from June 2019 to August 2019 for a time period of three months.

Study population: The study population was selected from among patients attending to the Surgery Department of Chettinad Academy of Research and Education.

Study design and patients: This was a case control study with 60 diabetic foot ulcers patients of both sexes, aged between 30–70 years, who attended the Surgery OPD of Chettinad Hospital and Research Institute (CHRI) and 60 age and sex matched patients with H/O Diabetes mellitus but without diabetic foot ulcer as controls.

Inclusion criteria: Subjects of both sexes admitted with C/O diabetic foot ulcers at the Department of Surgery, Chettinad Hospital and Research Institute, Kelambakkam.

Exclusion criteria

1. Those patients with H/O intake of anti platelet drugs, lipid lowering drugs.
2. History of prior treatment with corticosteroids.
3. Patients with hematological or euplastic disorders.
4. Those who had undergone recent surgeries, hyperthyroidism, cancer, pregnancy.

Laboratory, anthropometric and clinical data collection

After obtaining consent, DFU subjects were grouped based on the Wagner's classification of diabetic foot with the help of the treating plastic surgeon. Height and weight were

© 2020 The Authors

measured and BMI was calculated using the formula: Weight in Kg/(Height in Meters)^2. 5 ml of blood was collected from each patient in sodium citrate tube of light blue colored and violet-colored vacutainers. Fibrinogen, PT and APTT, and HbA1c were measured on the same day. Samples were stored at –20°C for the duration of the study.

Fibrinogen was measured by the turbidimetric method in a CA-50 machine in the pathology laboratory. Reference interval – 246–306 mg/dl.

Prothrombin Time (PT) and activated partial Thromboplastin Time (APTT) were measured in Pathology Laboratory using the photo optical method in a Sysmex CA-50 machine. The reference interval for prothrombin time is 11–12 seconds and for APPT it is 26–40 seconds. Hemoglobin levels were measured using cyan-met hemoglobin method in the Beckman Coulter Automated Machine in the pathology laboratory.

HbA1c was estimated in the D-10 machine based on the high performance liquid chromatographic (HPLC) principle in the biochemistry laboratory. Reference interval: 4.6–5.4%.

The DFU subjects are classified based on the severity of diabetic foot is by Wagner's classification which is as follows:

Class 1: Foot at risk;
Class 2: Superficial ulcers;
Class 3: Deep ulcers without osteitis;
Class 4: Deep ulcers with osteitis;
Class 5: Localized gangrene;
Class 6: Extensive gangrene

Statistical analysis

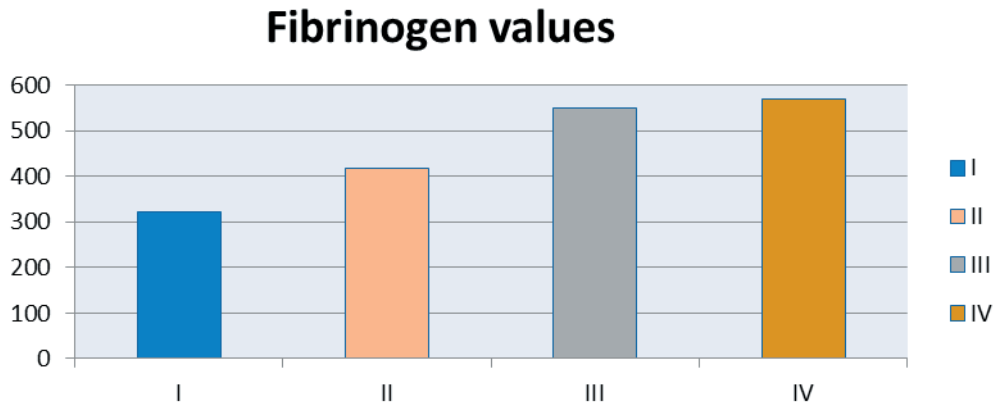
Sample size was calculated under the guidance of a statistician. SPSS software version 21 was used to analyze the results. SPSS version 21 was utilized for the statistical analysis. Paired t-test was used to compare the variables between the cases and controls. Pearson's correlation analysis was done to analyze the correlation between HbA1c and fibrinogen, PT and APTT. The diagnostic performance was assessed using receiver operating characteristic curve (ROC curve). P value less than 0.05 was considered to be significant.

Table 1: Paired T test done to find out the difference in the variables between the DFU cases and the normal healthy controls (p value<0.05 is significant).

Variables Demographic details	Cases		Control		p value
	Mean	SD	Mean	SD	
Age(Years)	56.85±10.8	10.8	54±10	10	0.14
Sex- Female (%)	23%		30%		—
Male (%)	67%		70%		—
Height(cm)	168.67±4.64	4.64	167.2	3.90	0.059
Weight(cm)	79.43	7.12	69.30	4.00	<0.0001*
BMI	27.8	1.51	24.63	0.8	<0.0001*
Duration of diabetes(years)	7.78	5.44	6.87	4.66	0.3
Laboratory parameters					
Hemoglobin (g/dl)	12.25	2.47	11.6	2.3	0.56
HbA1c (%)	9.5	2.7	6.7	2.5	0.05*
Prothrombin time (PT) (sec)	16.68	4.4	12.5	1.6	0.0001*
Activated Partial thromboplastin time (APTT) (sec)	36.16	7.07	34.45	7.3	0.24
Fibrinogen (mg/dl)	452.3	136.4	306.65	79.6	0.0001*

HbA1c –Glycated hemoglobin.

https://doi.org/10.46389/rjd-2020-1050
© 2020 The Authors



Fibrinogen levels (mg/dl) –Grade -1-320.56, Grade-II-417.25, GradeIII-548.99, Grade IV-570.5

Figure 1: Shows the mean value of fibrinogen (mg/dl) in the different grades of diabetic foot ulcers.

Results

Demographic variables and laboratory variables between the cases and controls were compared using paired test (Table 1). Among the cases, 12 belonged to Grade I group, 18 to Grade II, 25 to Grade III, and 5 to Grade IV group. The mean fibrinogen levels in Group I was 320.56±105.25, Group II was 417.25±82, Group III was 548.99±122.2, and Grade IV was 570.4±150.68 respectively (Fig. 1). The fibrinogen levels were found to be higher as the grade of DFU increases. The mean level of fibrinogen among cases was 452.3±136.4 and among controls was 306.5±79.6 respectively, and there was statistically significant difference between the two groups (p value = 0.0001). Prothrombin time (PT) showed mean value of 16.68±4.2 sec among cases and 12.5±1.6 sec among controls and was statistically significant (p value = 0.0001). APTT showed mean value of 36.2±7.07 sec among cases compared to 34±7.3 sec among controls but the difference was not statistically significant (p value = 0.24). (Fig. 2**)** depicts the levels of PT, APTT, and fibrinogen among DFU cases and controls. Pearson's correlation analysis between HbA1c and fibrinogen levels showed that there was significant positive correlation between HbA1c and fibrinogen levels among DFU patients with correlation coefficient (r value of 0.759) (Fig. 3). The correlation

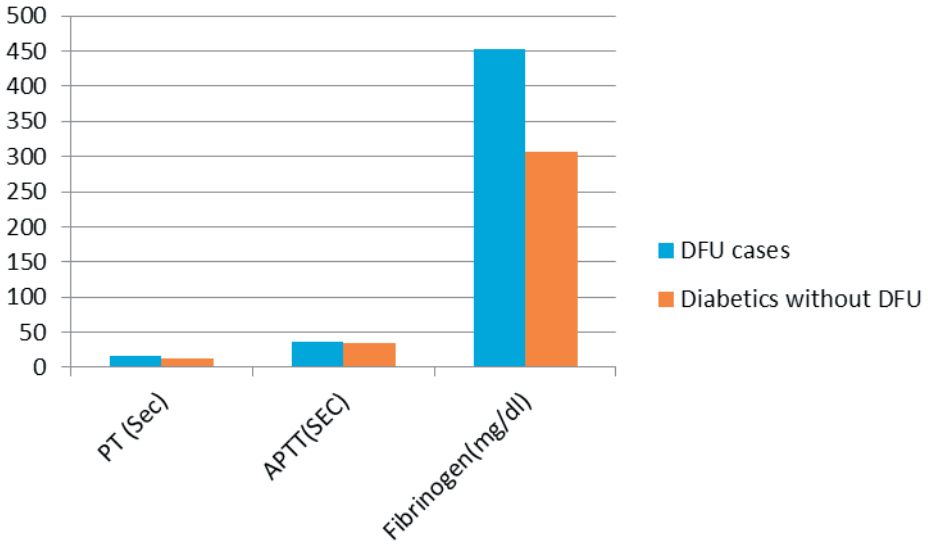


Figure 2: Shows the mean value of PT, APTT and fibrinogen among the DFU patients and diabetes patients without DFU.

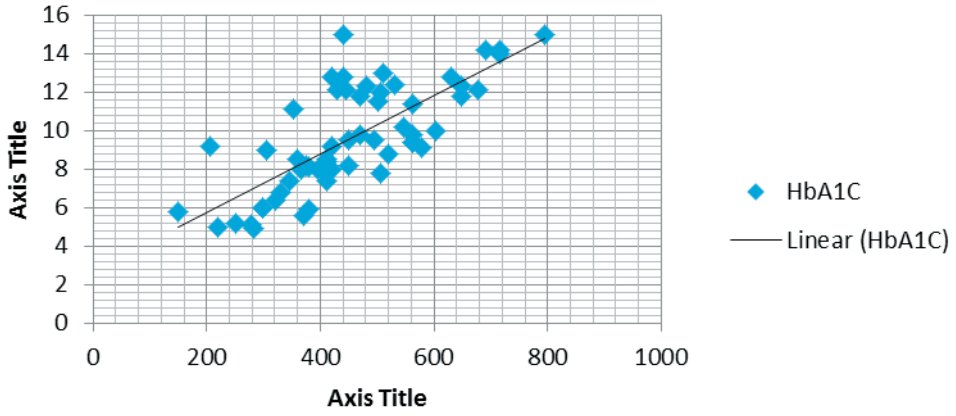


Figure 3: Scatter plot between HbA1c and fibrinogen levels among DFU patients.

Table 2: Shows the correlation analysis of variables (HbA1c and PT, HbA1c and APTT, HbA1c and Fibrinogen) (p value <0.05 is significant).

Correlation analysis	PT (r value)	p value	APTT (r value)	P value	Fibrinogen (r value)	p value
HbA1c	0.184	0.162	0.017*	0.89	0.75	0.00*

PT-Prothrombin Time, APTT-Activated Partial Thromboplastin time, HbA1c-Glycated Hemoglobin.

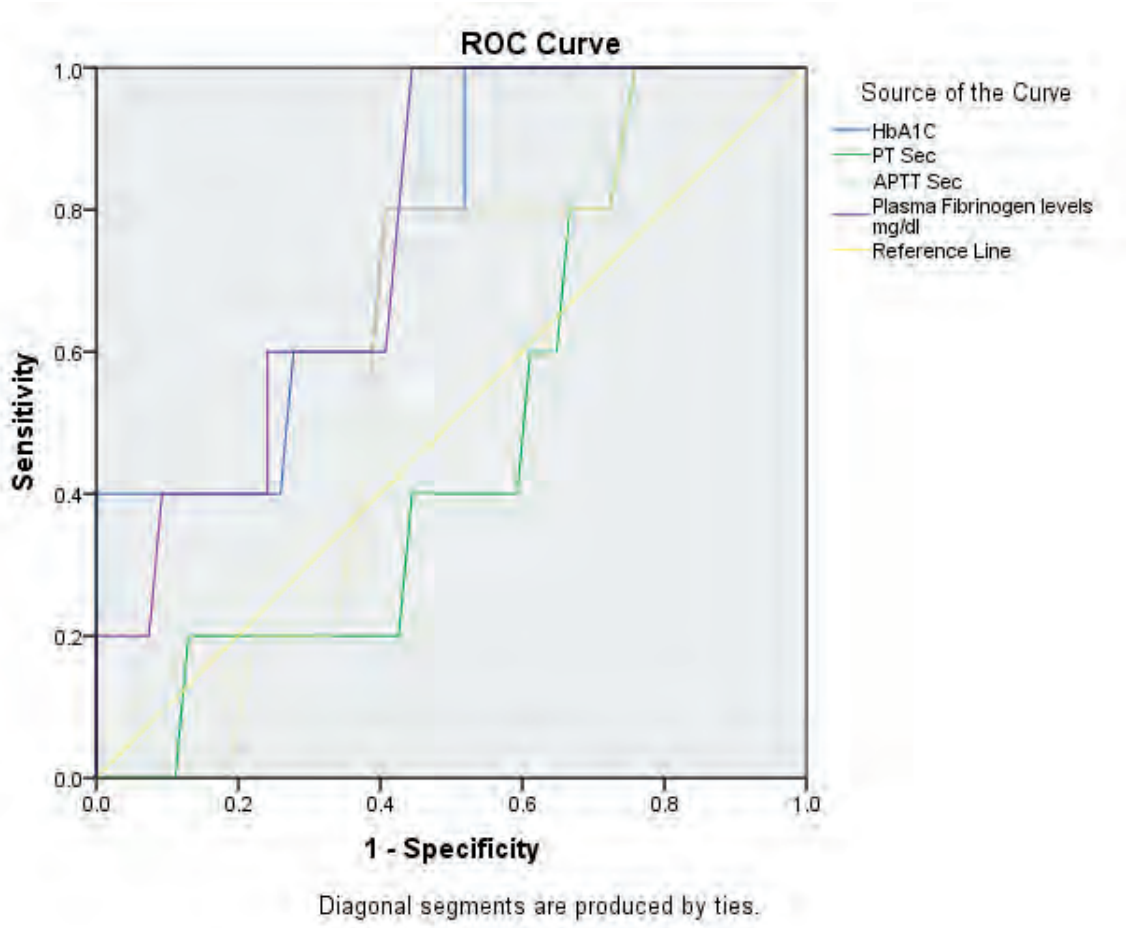


Figure 4: ROC curve of fibrinogen showing its ability to diagnose diabetic foot ulcers (DFU).

https://doi.org/10.46389/rjd-2020-1050
© 2020 The Authors

between HbA1c and PT and APTT was not found to be significant (r = 0.184, p value = 0.162), (r = 0.017, p value = 0.9). The correlation coefficients between HbA1c and fibrinogen values in Grade I was (r value = 0.561) (p value = 0.072), Grade II (r value = 0.704) (p value = 0.00), Grade III (r value = 0.860) (p value = 0.00), Grade IV (r value = 0.413) (p value = 0.49). Correlation analysis between HbA1c and PT showed a negative correlation coefficient of r = 0.18, HbA1c and APTT showed a correlation coefficient of r = 0.017 but was not statistically significant (Table 2). ROC analysis shows that the area under the curve for HbA1c fibrinogen is 0.758 and 0.760 respectively, but PT and APTT doesn't show much significance (Fig. 4).

Discussion

Diabetes is a disease characterized by enhanced activation of the coagulation profile and disturbances in the integrity of the vascular epithelium. Glycated hemoglobin (HbA1c) gives us an idea of the diabetic control of patients for the last three months. The mean value of HbA1c was found to be higher among diabetic foot ulcers patients (9.5) when compared to the diabetic controls with. Glycated hemoglobin was found to have a positive correlation with plasma fibrinogen levels. This finding is in accordance to the study done by Madan *et al.* among Japanese school children, who found out a significant association between fibrinogen and HbA1c [12].

The correlation between glycemic control and fibrinogen levels in this study could be because glycated fibrinogen is less susceptible to degradation by plasmin and the relative insulin deficiency in Type-II Diabetes mellitus causes an imbalance in the protein synthesis, leading to a 29% decrease in albumin synthesis and 50% increase in synthesis of fibrinogen synthesis [13].

Our results have shown that the fibrinogen levels increased with the increase in the grade of DFU, which was on par with the studies done by Rattan et al., and Li et al., who also found out that fibrinogen levels increased with increase in severity of DFU [14, 15]. Weigelt et al., showed that there was no significant difference in the levels of fibrinogen between the different grades of DFU [16].

Fibrinogen plays an important role in thrombosis, development of subclinical atherosclerosis, formation of fibrin clot. The coagulation cascade is activated by hyperglycemia, leading to hyperfibrinogenemia. This leads to an increase in fibrinogen degradation products, which will inturn stimulate the synthesis of fibrinogen from the liver. The various mechanism by which fibrinogen has been found to promote thrombosis are that hyperfibrinogenemia increases plasma viscosity, induces aggregation of RBC, causes platelet aggregation, forms fibrin and fibrinogen degradation products (FDPs) which in turn bind LDL and sequester more fibrinogen which in turn stimulate smooth cell proliferation and migration. [17, 18]. ROC (Receiver operating curve) curve shows that fibrinogen also shows higher specificity and sensitivity to diagnose diabetic foot ulcers when compared with PT and APTT.

Prothrombin time was found to be higher than the reference interval among diabetic foot ulcers patients. APTT levels were found to be within normal limits. But when compared with the control group, the levels were lower than the cases and were found to be statistically significant. Studies done by Alao O et al are on par with this study showing prolonged PT and APTT levels among diabetic foot ulcers patients. Our study did not show much correlation between HbA1c and PT, APTT [19]. Studies by Collier et al. have shown that the levels of PT and APTT among diabetic foot ulcers patients were within the normal reference range but were lower when compared with the control group [20].

Diabetes is a pro-coagulant state. The variations among the coagulation profile have only been partially understood. Plasma fibrinogen levels were significantly elevated among diabetic foot ulcers patients, and gradually increased as the ulcer grade increased, but prothrombin time, activated partial thromboplastin time showed no particular significance. More extensive studies are required to use it as a marker for assessing the severity of DFU.

© 2020 The Authors

Conclusions

This study was conducted among patients with diabetic foot ulcers to assess the correlation between HbA1c and plasma fibrinogen level, PT and APTT. We found that the fibrinogen levels increased with an increase in the grade of DFU. There was a significant positive correlation between HbA1c levels and fibrinogen among Grade 2 and Grade 3 classes of DFU. Prothrombin time also showed significant difference between the two groups. Fibrinogen and Prothrombin time were found to have a promising role in the diagnosis of diabetic foot ulcers. With further studies, we can utilize these markers as screening tools to assess hyper coagulable states of diabetes mellitus.

Acknowledgements

We thank the Chettinad management for giving us the opportunity to conduct this study.

Conflict of interest

The authors declare no conflict of interest.

Funding

This study was funded by ICMR-STS.

References

1. R.Sicree, Richard. Diabetes and impaired glucose tolerance. *Diabetes atlas*. 15–109, 2006.
2. Update of mortality attributable to diabetes for the IDF Diabetes Atlas: Estimates for the year 2013. *Diabetes research and clinical practice*. 109(3), 461–5, 2015.
3. Singh N, Armstrong DG, Lipsky BA. Preventing foot ulcers in patients with diabetes. JAMA. 293(2), 217–228, 2005.
4. Jabeen, F, Fawwad, A, Rizvi, H.A, Alvi, F. Role of platelet indices, glycemic control and hs-CRP in pathogenesis of vascular complications in type-2 diabetic patients. *Pakistan journal of medical sciences*. 29(1), 152, 2013.
5. Lipsky B.A, Berendt A.R, Cornia, P.B, Pile J.C, Peters E.J, Armstrong D.G, Deery et.al. Infectious Diseases Society of America clinical practice guideline for the diagnosis and treatment of diabetic foot infections. *Clinical infectious diseases*. 54(12), 132–173, 2012.
6. Armstrong DG, Lavery LA. Diabetic foot ulcers: prevention, diagnosis and classification. *American family physician*. 57(6), 1325, 1998.
7. Clayton W, Elasy TA. A review of the pathophysiology, classification, and treatment of foot ulcers in diabetic patients. *Clinical diabetes*: 27(2), 52–58, 2009.
8. Bowering CK. Diabetic foot ulcers. Pathophysiology, assessment, and therapy. *Canadian Family Physician*. 47, 1007–1016, 2001.
9. Kannel W.B, D'Agostino R.B, Wilson P.W, Belanger A.J, Gagnon D.R. Diabetes, fibrinogen, and risk of cardiovascular disease: the Framingham experience. *American heart journal*. 120(3), 672–676, 1990.
10. Knöbl P, Schernthaner G, Schnack C, Pietschmann P, Griesmacher A, Prager R, Müller M. Thrombogenic factors are related to urinary albumin excretion rate in type 1 (insulin-dependent) and type 2 (non-insulin-dependent) diabetic patients. *Diabetologia*. 36(10), 1045–1050, 1993.
11. Stec JJ, Silbershatz H, Tofler GH, Matheney TH, Sutherland P, Lipinska I, Massaro JM, et al. Association of fibrinogen with cardiovascular risk factors and cardiovascular disease in the Framingham Offspring Population. *Circulation*. 102(14), 1634–1638, 2000.
12. Fujii C, Sakakibara H, Kondo T, Yatsuya H, Tamakoshi K Toyoshima H. Plasma fibrinogen levels and cardiovascular risk factors in Japanese schoolchildren. *Journal of epidemiology*. 16(2), 64–70, 2006.
13. Bembde AS. A study of plasma fibrinogen level in type-2 diabetes mellitus and its relation to glycemic control. *Indian Journal of Hematology and Blood Transfusion*. 28, 105–108, 2012.
14. Rattan, R. and Nayak, D. High levels of plasma malondialdehyde, protein carbonyl, and fibrinogen have prognostic potential to predict poor outcomes in patients with diabetic foot wounds: a preliminary communication. *The international journal of lower extremity wounds*, 7(4), 198-203, 2008
15. Li XH, Guan LY, Lin H.Y, Wang SH, Cao YQ, Jiang XY, Wang YB. Fibrinogen: a marker in predicting diabetic foot ulcer severity. *Journal of diabetes research*. 1, 2016.
16. Weigelt C, Rose B, Poschen U, Ziegler D, Friese G, Kempf K, Koenig W, Martin S. Herder C, Immune mediators in patients with acute diabetic foot syndrome. *Diabetes care*: 32(8), 1491–1496, 2009.
17. De Feo P, Gaisano M.G, Haymond MW. Differential effects of insulin deficiency on albumin and fibrinogen synthesis in humans. *The Journal of clinical investigation*. 88(3), 833–840, 1991.
18. Carmassi F, Morale M, Puccetti R, De Negri F, Monzani F, Navalesi R, Mariani G. Coagulation and fibrinolytic system impairment in insulin dependent diabetes mellitus. *Thrombosis research*. 67(6), 643–654, 1992.
19. Alao O, Damulak D, Joseph D, Puepet F. Haemostatic profile of patients with type 2 diabetes mellitus in Northern Nigeria. *The Internet Journal of Endocrinology*. 6(1), 1–4, 2009.
20. Collier A, Rumley A, Rumley AG, Paterson JR, Leach JP, Lowe G.D, Small M. Free radical activity and hemostatic factors in NIDDM patients with and without microalbuminuria. *Diabetes*. 41(8), 909–913, 1992.

 https://doi.org/10.46389/rjd-2020-1050 © 2020 The Authors

https://doi.org/10.46389/rjd-2020-1051
www.rjdnmd.org

Rom J Diabetes Nutr Metab Dis
2020; volume 27, issue 4, pages 349-356

Original Research

Decrease of plasma TNF-α and CRP levels in response to post-exhaust resistance training and vitamin D supplementation in overweight healthy women

Narges Kallantar[1], Hoseyn Fatolahi[2]*

[1] Department of Exercise Physiology, Central Tehran Branch, Islamic Azad University, Tehran, Iran
[2] Department of Physical Education, Pardis Branch, Islamic Azad University, Pardis, Iran

*Correspondence to: Hoseyn Fatolahi, Department of Physical Education, Pardis Branch, Islamic Azad University, Pardis, Iran.
E-mail: hoseyn.fatolahi@pardisiau.ac.ir

Received: 15 May 2020 / Accepted: 30 August 2020

Abstract

Introduction: Diabetes are the most common diseases in the world and is related to nutritional status and lifestyle. The purpose of this study was to investigate the simultaneous effect of resistance training and vitamin D supplementation on plasma CRP and tumor necrosis factor alpha (TNF-α) levels in overweight healthy women. **Method:** The participants were randomly divided into four groups including: (1) placebo, (2) resistance training (RT), (3) vitamin D, and (4) RT + vitamin D. Interventions were performed for 8 weeks (3 days per week), consisting of resistance training protocol (60% 1-RM) and taking vitamin D (1000 IU/day). Fasting blood samples were collected 48 hours before and after the interventions. **Result:** A significant decrease in CRP was reported among the studied groups (p=0.001, F=11.4). These changes showed a difference between RT+ vitamin D compared to other groups. The CRP values of the RTand vitamin D groups were also significantly lower than the placebo group (p=0.03). TNF-α was significantly decreased among the studied groups (p=0.003, F = 5.4). These changes showed a difference between the RT + vitamin D group compared to other groups. TNF-α was significantly lower in the RTand vitamin D groups than in the placebo group (p=0.03). **Conclusion:** The findings of this study confirm that adaptation to resistance training, if combined with vitamin D intake, has significant effects on decreasing inflammatory biomarkers at rest. In addition, TNF-α alteration appears to be less effective than resistance training, which may be due to eccentric contractions caused by resistance training.

Keywords: Exercise rehabilitation, Inflammation, Post-exhaust, Resistance Training, Supplementation.

Introduction

Obesity, poor nutrition and sedentary life style, and systemic inflammation are important communication for diseases and the most important reason is the secretion of cytokines and inflammatory agents caused by adipose tissue [1, 2]. Researchers are looking for the best and least risky ways to control overweight and reduce adipose tissue. Nutritional controls, medications, and regular exercise or combinations of these techniques are highly recommended [2]. Obesity is associated with chronic inflammation, which is characterized by the infiltration of immune cells into adipocytes that contribute to the release of cytokines which induce inflammation, which, in turn, leads to inflammatory signaling pathways [1]. Cytokines are a group of low-molecular-weight regulatory proteins secreted by adipose tissue, white blood cells, and other types of body cells. Cytokines generally act as intracellular messenger molecules that initiate specific biological activities after binding to a receptor on the target cells [3–5].

Cytokines may require other physiological functions to play a role in inflammatory responses especially if they are secreted from adipose tissue [3, 4, 6, 7]. Cytokine is a generic title and is specifically called adipokine if expressed by adipose tissue. Most evidence suggests that the presence of mild inflammation in obesity is

© 2020 The Authors. Romanian Journal of Diabetes, Nutrition and Metabolic Diseases published by Sanatatea Press Group on behalf of the Romanian Society of Diabetes Nutrition and Metabolic Diseases. This is an open access article under the terms of the Creative Commons Attribution License (CC-BY-NC-ND 3.0).

associated with changes in the levels of several circulating biomarkers, such as increases in plasma C-reactive protein (CRP) and tumor necrosis factor alpha (TNF-α) [3–7].

Regular exercise is one of the methods recommended to reduce inflammation biomarkers including TNF-α and CRP [8–9]. These findings were established in overweight and metabolic syndrome [10]. Among all kinds of exercises, resistance training has been shown to improve CRP levels, although no integrated information is available on TNF-α change [11]. Obese people have been reported to be more prone to vitamin D deficiency and inflammation [12–14]. Inflammation and metabolic syndrome develop in people who have symptoms of obesity, diabetes, inflammation, hypertension and dyslipidemia, which are reduced by vitamin D supplementation [12–14]. Vitamin D is one of the types of fat-soluble vitamins called calciferol. The ergocalciferol (vitamin D2) and cholecalciferol (vitamin D3) are its metabolites [12–14].

In summary, some studies have investigated the concomitant effects of physical activity and vitamin D supplementation on various domains of exercise physiology [15], but few studies have investigated the concurrent medication effects of resistance exercise training and vitamin D on inflammatory markers in overweight healthy women. Differences in the methodology of these limited studies also, make it difficult to come up with an integrated viewpoint. There is not a clear finding about the effective dose of vitamin D [12]. In addition, there is no specific and effective method for reducing inflammatory cytokines based on the specificity principle of exercise training [11]. It has to be noted that the tendency to perform resistance training in indoor spaces has increased and this has led to the deprivation of sunlight. It is also important to examine this issue in women's health because of their important role in family and community. Therefore, the aim of this study was to evaluate the effect of vitamin D supplementation with resistance training on inflammatory markers in overweight healthy women.

Materials and Methods

A total of 103 university student women were assessed for eligibility. Forty sedentary overweight healthy women were equally divided into four groups including: (1) placebo, (2) vitamin D, (3) resistance training (RT), and (4) resistance exercise + vitamin D (Table 1). There were no serious side effects associated with regular exercise, and no one withdrew due to side effects. The participants were overweight (Table 1) and their vitamin D levels were normal in the lower extremities (Table 1) (reference range minimum for health bone: 20–32 ng/ml), so they needed to be prescribed daily vitamin D, according to doctor's prescription. Body mass index was calculated in kg/m^2 using BMI equation through measuring height (Seca 213, Germany recorded

Table 1: Anthropometric characteristics of the subjects and measured variables among the studied groups. Data are presented by mean and standard deviation.

Groups	RT + vitamin D		resistance training (RT)		vitamin D		placebo	
Time	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Age	27.9±3.8	—	29.2±3.3	—	29.2±3.2	—	27.4±1.4	—
Height	162.2±4.8	—	161.4±4.08	—	162.2±3.4	—	163.8±6.1	—
Weight (kg)	71.4±3.7	70.4±4.05	70.9±4.2	70.2±5.3	69.1±4.8	68.9±3.1	72.6±6.1	72.4±6.2
BMI (kg/m^2)	27.9±1.3	27.5±1.4	27.2±1.8	27±2.4	26.2±0.7	26.2±0.7	27.4±1.5	26.9±1.5
TNFα (ng/ml)	0.96±0.1	0.83±0.05	0.91±0.1	0.8±0.03	0.95±0.1	0.91±0.08	0.89±0.2	0.87±0.1
CRP (ng/ml)	3.01±0.82	1.96±0.71	2.93±0.5	2.61±0.63	2.99±0.62	2.67±0.62	2.98±0.52	2.91±0.46
25-OH-Vit D (ng/ml)	23.7±1.3	28.2±1.6	22.9±1.2	24.7±1.8	23.6±1.7	27.2±1.5	22.8±1.4	23.4±1.3

https://doi.org/10.46389/rjd-2020-1051
© 2020 The Authors

to the nearest 0.1 cm.) and weight (SECA Digital Scale Model 727: with precision of 2 g).

The participants were provided with information and knowledge on how to conduct the research stages. A questionnaire was used to collect information about the physical activity and health of the participants. Consent form was given to the participants. The introductory program was held two weeks prior to the start of intervention to attend the gym. Anthropometric measurements were taken at a separate session. The participants were familiarized with exercise protocol, vitamin D supplementation, and blood sampling timing.

Resistance training protocol (eight weeks and three sessions per week) was performed by 50–60% 1-RM (three courses, 10 repeats, and a 2-minute rest between each movement). Exercises training included engaging chest, triceps and shoulder (first session), abdomen and dorsalis (second session), and leg muscles (third session). Major muscles using multiple joint movements at first and single muscle movements were applied at the end of each session (post exhausting method). Vitamin D supplementation groups (produced by the United Kingdom-Health Aid Company) received 1000 IU/day, which was administered for eight weeks. Blood samples were collected 48 hours before and after the intervention. Fasting blood samples were collected at the laboratory at 8 a.m. Resistance exercises were held at 5 p.m. Vitamin D was prescribed daily along with lunch.

Fasting blood samples were discharged into tubes containing EDTA. The samples were centrifuged at 4°C for 15 minutes at a speed of 10,000 rpm. Isolated plasma was stored at –70°C and used to measure the research variables. The plasma CRP and TNF-α levels were measured using a particle enhanced turbidimetris assay (Roche, Germany) and an enzyme-linked immune sorbent assay (ELISA. eBioscience, Austria) respectively, according to the manufacturer instruction. The LIAISON 25-OH Vitamin D TOTAL Assay (DiaSorin) was used to measure plasma concentration of 25-hydroxyvitamin D (25(OH)D).

The Kolmogorov-Smirnov test was used to determine the normal distribution of data. The ANCOVA was used to test the research hypotheses after determining the pre-test and post-test differences. The sphericity of the data was confirmed by performing variance analysis (Mauchly's Test of Sphericity). Tukey's*post hoc* test was used for between-group comparisons. Statistical analyses were performed using SPSS 21 computer software at the significant level $p \leq 0.05$.

Results

The research data and measured characteristics of the participants, including age, height, weight, and BMI, are presented in Table 1.

The significant differences for vitamin D levels were reported among the studied groups after interventions (p=0.03, F=8.62). These changes showed a difference between the vitamin D and RT + vitamin D groups compared to the RT (p=0.03) and control (p=0.02) groups. The significant differences forplasma CRP levelswere reported amongthe studied groups (p=0.001, F=11.4). These changes showed a difference between the RT+ vitamin D compared to theRT group (P=0.03), RT + vitamin D compared tothe vitamin D group (p=0.001), RT + vitamin D compared to the placebo group (p=0.001) and RTcompared to the placebo group (p=0.03). In addition, plasma TNF-αlevels were significantly different among the studied groups (p=0.003, F=5.4). These changes showed a significant difference between the RT + vitamin D group compared to the vitamin D group (p = 0.03), RT + vitamin D group compared to the placebo group (p = 0.003) and RTcompared tothe placebo group (p = 0.03). Despite some reductions for weight and BMI variables, there was no significant difference amongthe studied groups.

Discussion

The aim of this study was to evaluate the effect of vitamin D supplementation with resistance training on plasma TNF-α and CRP levels in overweight healthy women. In summary, in the present study, combination of regular resistance training with vitamin D supplementation significantly reduced the CRP and TNF-α rest levels.

© 2020 The Authors

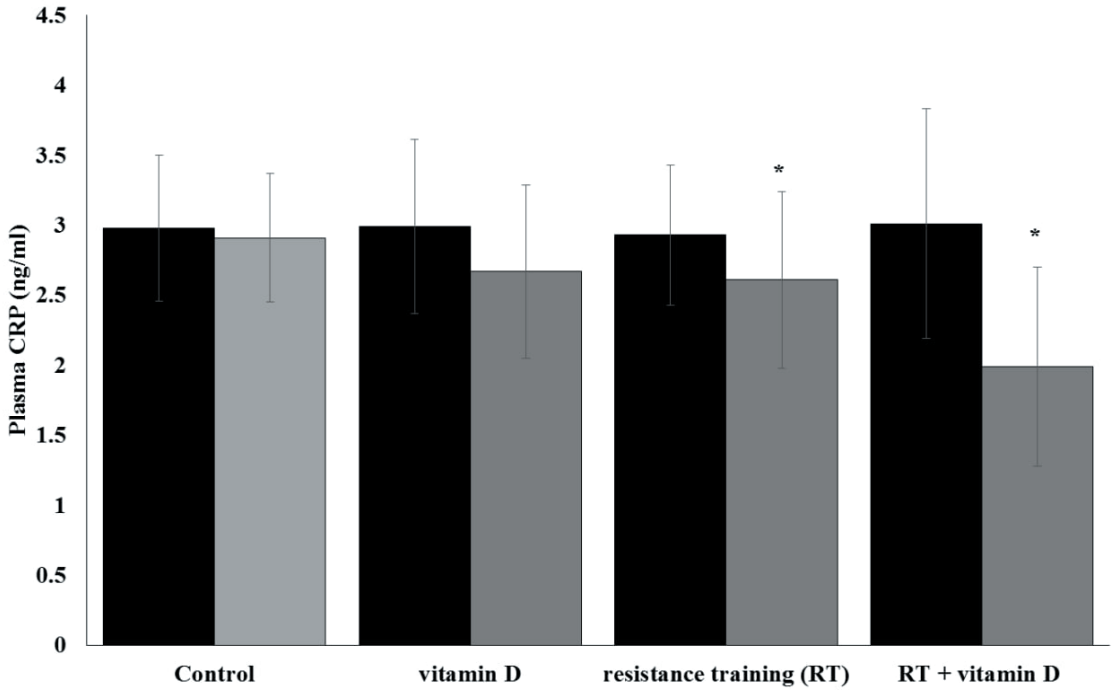


Figure 1: Plasma CRP level changes among the studied groups. Data are presented by mean and standard deviation. *Significant difference with other groups.

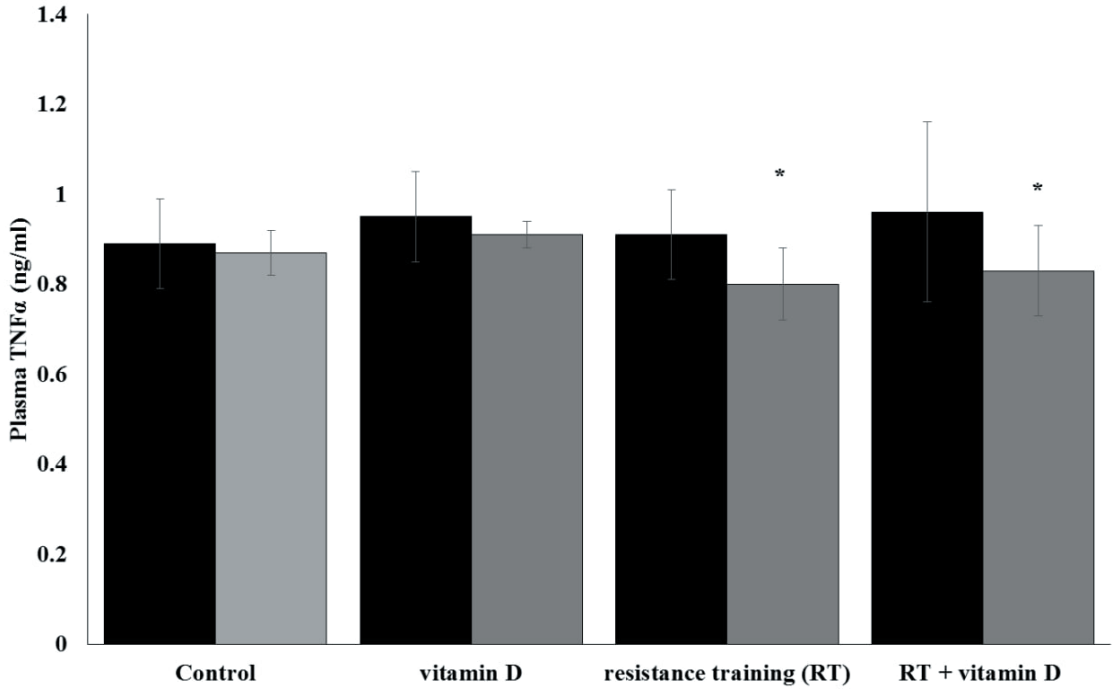


Figure 2: Plasma TNFα level changes among the studied groups. Data are presented by mean and standard deviation. *Significant difference with other groups.

The effect on the exercise training groups was also greater than the vitamin D group. However, the combination of resistance training and vitamin D showed a high decrease in plasma CRP and TNF-α rest levels.

Several mechanisms have been proposed to elucidate the inverse relationship between obesity and vitamin D that center around inflammation and include the role of adipokines, epigenetics, calcium, and adipose tissue type [16]. Simultaneous effects of vitamin D and calcium on hsCRP and TNF-α reduction in metabolic syndromehave been confirmed [17]. The association between calcium and vitamin D has been an

 https://doi.org/10.46389/rjd-2020-1051 © 2020 The Authors

important factor in improving glucose levels in obese and overweight women who have ovarian problems and vitamin D deficiency [18]. It has to be noted that exercise increases calcium pump function in the sarcoplasmic reticulum and is one of the possible pathways to regulating glucose entry into the muscle cell during exercise [19], whereas increased TNF-α is one of the major factors of fatigue caused by resistance training [5]. However, this is likely reduced by adaptation to resistance training.

Measuring the variables 48 hours after training is also very effective. In many studies, acute responses to exercise increased inflammatory responses, whereas the present study aimed to evaluate the adaptation with interventions. Since one of the major sources of inflammatory cytokines is adipose tissue, vitamin D and resistance training may reduce the concentration of inflammatory markers by reducing the body fat. Although weight and BMI in the present study did not show a significant decrease, it could also be due to increased or maintained muscle mass resulting from resistance training. However, some studies have reported changes in inflammatory markers independent of adipose tissue changes [20]. Low intensity exercise with 50% strength training at four sessions per week in postmenopausal and obese women decreased weight and waist circumference but did not significantly change CRP and TNF-α levels [21].

It is likely that daily food intake interventions can also be effective on inflammation. Due to the variety of research methods, the effect of daily foods on the production of inflammatory markers remains unknown [22]. There is limited information on the relationship between vitamin D receptors in metabolic syndrome and inflammation. However, it has been suggested that Fokl-type receptors are associated with moderate inflammation and insulin resistance [23]. It has also been reported that vitamin D levels affect exercise performance through association with iron levels [24]. Therefore, the presence of female participants in the study who are likely to have lower sports performance and lower iron levels compared to menwas effective in the results of this study.

The effect of vitamin D on reducing inflammation has also been identified, but the mechanisms of this effect are not fully understood. Vitamin D has been reported to have a modest effect on reducing inflammation in cardiovascular patients [25]. Meta-analysis studies have not conclusively confirmed the therapeutic effect of vitamin D alone on improving metabolic syndrome and inflammatory biomarkers [14]. Other articles that have even used a one-year course of vitamin D treatment have shown some inadequate efficacy of vitamin D treatment alone, despite improving inflammatory markers such as TNF-α, CRP, and IL-6 [26]. Inflammatory markers of TNF-α, CRP, and IL-6, unlike metabolic syndrome indices, did not change significantly in response to low doses of vitamin D [27]. Patients who also used dyslipidemia medication had improved vitamin D levels and subsequently metabolic syndrome indices, but no significant change in TNF-α levels was observed [28]. Probably one of the missing factors in this area is the combination of vitamin D intake with regular physical activity. An increase in vitamin D has been observed in response to a long period of regular exercise and lifestyle modification [29].

This topic has been well demonstrated with resistance training in obese and overweight adults [30, 31]. Adaptation to combined physical activity reduces CRP and TNF-α in healthy and active individuals [32]. Physical activity, especially if exposed to the sunlight, can be effective in boosting vitamin D that leads to improve athletic performance [33]. Therefore, reducing inflammation may require higher doses of vitamin D and complementary therapies such as regular physical activity. For example, changes in CRP have been observed at high doses and longer periods [34]. As mentioned, another reason for the improvement of inflammatory markers in the present study was the use of resistance training as a supplement to vitamin D. Regarding the health status of participants, it is very important in methodological studies as it was mentioned.

The exercise training and vitamin D supplementation have better effects on healthy groups. It has been shown that lean people have better improvement in low-dose (400–800 IU/d), moderate dose (1600–2400IU/d) and high dose of vitamin D (3200–4800 IU) compared to

© 2020 The Authors

obese. This may be due to differences in baseline vitamin D levels [35]. Meta-analysis studies have reported that vitamin D intake in overweight or obese groups may not have a significant effect on CRP and TNF-α [13]. Therefore, presence of overweight healthy women may be one of the reasons for the improvement of inflammatory markers in the present study. Inflammatory biomarkers may have a better response to exercise interventions in healthy individuals than patients, especially for CRP [36]. Probably one of the possible reasons for the better response of CRP to TNF-α in the present study, which has been reported in other studies, is the effect of eccentric contractions induced by resistance training on the increase of TNF-α [5]. Therefore, attention to this issue is especially important in weight loss in order to maintain muscle mass. However, this condition is improved by adaptation to exercise.

A comparison between aerobic and resistance training and their effects on inflammation in metabolic syndrome has been investigated. Some studies have suggested that the effect of aerobic activity on inflammatory biomarkers reduction including CRP and TNF-α is better than resistance training because of its direct effect on adipose tissue lipolysis [37]. However, TNF-α decreased significantly in response to aerobic training and CRP in response to both aerobic training and combination of resistance training and flexibility training [37]. In support of these findings, it has been reported that changes in TNF-α levels and adipose tissue in response to aerobic exercise and CRP level in response to resistance training were further reduced [38]. As noted, TNF-α changes in response to resistance training may be due to the eccentric contractions and differences in the source of secretion. Therefore, it seems necessary to incorporate resistance training or more intense training than the pattern of aerobic exercise to complete adaptations from exercise training. It is also important to use resistance training to stimulate intramuscular signaling pathways by stimulating intramuscular calcium pumps and simultaneously reducing adipose tissue and increasing or maintaining muscle mass [19]. However, it has been shown that the incorporation of resistance training specifically has maximized the effect of regular aerobic exercise on reducing inflammation [36].

Conclusion

There is a significant relationship between weight control, vitamin D intake and inflammation reduction. However, combining regular physical activity with these interventions increases the effectiveness on health. In fact, weight loss is associated with changes in the levels of inflammatory biomarkers, including TNF-α and CRP. It should be borne in mind that various studies have examined these multiple approaches on different variables that each biomarker has its own signaling pathways. Each biomarker according to the secretion source can have a unique signaling pathway. It is likely that each signaling pathway will have an optimal response to some type of specific exercise. Therefore, considering different aspects of a particular variable and the specificity principle of training are the most important aspects of studies in the field of exercise physiology for future research.

Submission statement

The manuscript has not been published and is not under consideration for publication elsewhere.

Each authors' contributions

Narges Kallantar did Investigation, Methodology, Project administration, Resources, Software.

Hoseyn Fatolahi did Investigation, Methodology, Project administration, Resources, Software, Formal analysis, Conceptualization, Supervision, Data curation, Writing – original draft, Writing – review & editing.

Conflict of interest

The authors declare no conflict of interest.

https://doi.org/10.46389/rjd-2020-1051
© 2020 The Authors

Funding

None.

Ethical approval

The authors of this paper would like to express their thanks to all participants in this study. The experimental protocol in this study was approved by the ethics committee of Islamic Azad University, Central Tehran Branch (No. 10121436962029). The researchers' Ethics Committee initially approved the experimental procedures and study protocols, which were fully explained to all participants, and a written consent form was signed after having read and understood the details of the experiments. The research was also conducted in accordance with the principles stated in the Declaration of Helsinki.

Acknowledgments

The authors of this paper would like to express their thanks to Histogenotech research center (www.histogene.ir) for their critical comments during the project.

References

1. Ellulu MS, Patimah I, Khaza'ai H, Rahmat A, Abed Y. Obesity and inflammation: the linking mechanism and the complications. *Archives of medical science: AMS*. 13(4):851–863, 2017.
2. Tseng YH, Cypess AM, Kahn CR. Cellular bioenergetics as a target for obesity therapy. *Nature reviews Drug discovery*. 9(6):465–482, 2010.
3. Sproston NR, Ashworth JJ. Role of C-Reactive Protein at Sites of Inflammation and Infection. *Frontiers in immunology*. 9:754, 2018.
4. Wang B, Trayhurn P. Acute and prolonged effects of TNF-alpha on the expression and secretion of inflammation-related adipokines by human adipocytes differentiated in culture. *Pflugers Archiv: European journal of physiology*. 452(4):418–427, 2006.
5. Wan JJ, Qin Z, Wang PY, Sun Y, Liu X. Muscle fatigue: general understanding and treatment. *Experimental & molecular medicine*.49(10):e384, 2017.
6. Montecucco F, Mach F. New evidences for C-reactive protein (CRP) deposits in the arterial intima as a cardiovascular risk factor. *Clinical interventions in aging*. 3(2):341–349, 2008.
7. Osman R, L'Allier PL, Elgharib N, Tardif JC. Critical appraisal of C-reactive protein throughout the spectrum of cardiovascular disease. *Vascular health and risk management*. 2(3):221–237, 2006.
8. Pearson MJ, Mungovan SF, Smart NA. Effect of aerobic and resistance training on inflammatory markers in heart failure patients: systematic review and meta-analysis. *Heart failure reviews*. 23(2):209–223, 2018.
9. Goldhammer E, Tanchilevitch A, Maor I, Beniamini Y, Rosenschein U, Sagiv M. Exercise training modulates cytokines activity in coronary heart disease patients. *International journal of cardiology*. 100(1):93–99, 2005.
10. Hayashino Y, Jackson JL, Hirata T, et al. Effects of exercise on C-reactive protein, inflammatory cytokine and adipokine in patients with type 2 diabetes: a meta-analysis of randomized controlled trials. *Metabolism: clinical and experimental*. 63(3):431–440, 2014.
11. de Salles BF, Simao R, Fleck SJ, Dias I, Kraemer-Aguiar LG, Bouskela E. Effects of resistance training on cytokines. *International journal of sports medicine*.31(7):441–450, 2010.
12. Dinca M, Serban MC, Sahebkar A, et al. Does vitamin D supplementation alter plasma adipokines concentrations? A systematic review and meta-analysis of randomized controlled trials. *Pharmacological research*. 107:360–371, 2016.
13. Jamka M, Wozniewicz M, Walkowiak J, Bogdanski P, Jeszka J, Stelmach-Mardas M. The effect of vitamin D supplementation on selected inflammatory biomarkers in obese and overweight subjects: a systematic review with meta-analysis. *European journal of nutrition*. 55(6):2163–2176, 2016.
14. Zuk A, Fitzpatrick T, Rosella LC. Effect of Vitamin D3 Supplementation on Inflammatory Markers and Glycemic Measures among Overweight or Obese Adults: A Systematic Review of Randomized Controlled Trials. *PloS one*. 11(4):e0154215, 2016.
15. Antoniak AE, Greig CA. The effect of combined resistance exercise training and vitamin D3 supplementation on musculoskeletal health and function in older adults: a systematic review and meta-analysis. *BMJ open*. 7(7):e014619, 2017.
16. Mehmood ZH, Papandreou D. An Updated Mini Review of Vitamin D and Obesity: Adipogenesis and Inflammation State. *Open access Macedonian journal of medical sciences*. 4(3):526–532, 2016.
17. Tabesh M, Azadbakht L, Faghihimani E, Tabesh M, Esmaillzadeh A. Calcium-vitamin D cosupplementation influences circulating inflammatory biomarkers and adipocytokines in vitamin D-insufficient diabetics: a randomized controlled clinical trial. *The Journal of clinical endocrinology and metabolism*. 99(12):E2485–2493, 2014.
18. Asemi Z, Foroozanfard F, Hashemi T, Bahmani F, Jamilian M, Esmaillzadeh A. Calcium plus vitamin D supplementation affects glucose metabolism and lipid concentrations in overweight and obese vitamin D deficient women with polycystic ovary syndrome. *Clinical nutrition*. 34(4):586–592, 2015.
19. Pagnotti GM, Styner M, Uzer G, et al. Combating osteoporosis and obesity with exercise: leveraging cell mechanosensitivity. *Nature reviews Endocrinology*. 15(6):339–355, 2019.
20. Thong FS, Hudson R, Ross R, Janssen I, Graham TE. Plasma leptin in moderately obese men: independent effects of weight loss and aerobic exercise. *American journal of physiology Endocrinology and metabolism*. 279(2):E307–313, 2000.
21. Arsenault BJ, Cote M, Cartier A, et al. Effect of exercise training on cardiometabolic risk markers among sedentary, but

metabolically healthy overweight or obese post-menopausal women with elevated blood pressure. *Atherosclerosis*. 207(2):530–533, 2009.
22. Labonte ME, Couture P, Richard C, Desroches S, Lamarche B. Impact of dairy products on biomarkers of inflammation: a systematic review of randomized controlled nutritional intervention studies in overweight and obese adults. *The American journal of clinical nutrition*. 97(4):706–717, 2013.
23. Mackawy AM, Badawi ME. Association of vitamin D and vitamin D receptor gene polymorphisms with chronic inflammation, insulin resistance and metabolic syndrome components in type 2 diabetic Egyptian patients. *Meta gene*. 2:540–556, 2014.
24. Orysiak J, Mazur-Rozycka J, Fitzgerald J, Starczewski M, Malczewska-Lenczowska J, Busko K. Vitamin D status and its relation to exercise performance and iron status in young ice hockey players. *PloS one*.13(4):e0195284, 2018.
25. Rodriguez AJ, Mousa A, Ebeling PR, Scott D, de Courten B. Effects of vitamin D supplementation on inflammatory markers in heart failure: a systematic review and meta-analysis of randomized controlled trials. *Scientific reports*. 8(1):1169, 2018.
26. Beilfuss J, Berg V, Sneve M, Jorde R, Kamycheva E. Effects of a 1-year supplementation with cholecalciferol on interleukin-6, tumor necrosis factor-alpha and insulin resistance in overweight and obese subjects. *Cytokine*. 60(3):870–874, 2012.
27. Belenchia AM, Tosh AK, Hillman LS, Peterson CA. Correcting vitamin D insufficiency improves insulin sensitivity in obese adolescents: a randomized controlled trial. *The American journal of clinical nutrition*. 97(4):774–781, 2013.
28. Beltowski J, Atanassova P, Chaldakov GN, Jamroz-Wisniewska A, Kula W, Rusek M. Opposite effects of pravastatin and atorvastatin on insulin sensitivity in the rat: role of vitamin D metabolites. *Atherosclerosis*. 219(2):526–531, 2011.
29. Kim HJ, Kang CK, Park H, Lee MG. Effects of vitamin D supplementation and circuit training on indices of obesity and insulin resistance in T2D and vitamin D deficient elderly women. *Journal of exercise nutrition & biochemistry*. 18(3):249–257, 2014.
30. Carrillo AE, Flynn MG, Pinkston C, et al. Vitamin D supplementation during exercise training does not alter inflammatory biomarkers in overweight and obese subjects. *European journal of applied physiology*. 112(8):3045–3052, 2012.
31. Carrillo AE, Flynn MG, Pinkston C, et al. Impact of vitamin D supplementation during a resistance training intervention on body composition, muscle function, and glucose tolerance in overweight and obese adults. *Clinical nutrition*. 32(3):375–381, 2013.
32. Cronin O, Keohane DM, Molloy MG, Shanahan F. The effect of exercise interventions on inflammatory biomarkers in healthy, physically inactive subjects: a systematic review. *QJM : monthly journal of the Association of Physicians*. 110(10):629–637, 2017.
33. Fernandes MR, Barreto WDRJ. Association between physical activity and vitamin D: A narrative literature review. *Revista da Associacao Medica Brasileira*.63(6):550–556, 2017.
34. Breslavsky A, Frand J, Matas Z, Boaz M, Barnea Z, Shargorodsky M. Effect of high doses of vitamin D on arterial properties, adiponectin, leptin and glucose homeostasis in type 2 diabetic patients. *Clinical nutrition*. 32(6):970–975, 2013.
35. Gallagher JC, Yalamanchili V, Smith LM. The effect of vitamin D supplementation on serum 25(OH)D in thin and obese women. *The Journal of steroid biochemistry and molecular biology*. 136:195–200, 2013.
36. Ihalainen JK, Schumann M, Eklund D, et al. Combined aerobic and resistance training decreases inflammation markers in healthy men. *Scandinavian journal of medicine & science in sports*. 28(1):40–47, 2018.
37. Kohut ML, McCann DA, Russell DW, et al. Aerobic exercise, but not flexibility/resistance exercise, reduces serum IL-18, CRP, and IL-6 independent of beta-blockers, BMI, and psychosocial factors in older adults. *Brain, behavior, and immunity*. 20(3):201–209, 2006.
38. Donges CE, Duffield R, Drinkwater EJ. Effects of resistance or aerobic exercise training on interleukin-6, C-reactive protein, and body composition. *Medicine and science in sports and exercise*. 42(2):304–313, 2010.

 https://doi.org/10.46389/rjd-2020-1051 © 2020 The Authors

https://doi.org/10.46389/rjd-2020-1052
www.rjdnmd.org

Rom J Diabetes Nutr Metab Dis
2020; volume 27, issue 4, pages 357-365

Original Research

The effect of Purslane and *Aquilaria malaccensis* on insulin-resistance and lipid peroxidation in High-fructose diet Rats

Samir Derouiche[1,2]*, Ouidad Degachi[1], Khaoula Gharbi[1]

[1] **Department of Cellular and Molecular Biology, Faculty of Natural and Life Sciences, El-Oued University, El Oued 39000, El Oued, Algeria**

[2] **Laboratory of Biodiversity and application of biotechnology in the agricultural field, Faculty of natural science sand life, University of El Oued, El-Oued 39000, Algeria**

*Correspondence to: Samir Derouiche, Department of Cellular and Molecular Biology, Faculty of Natural and Life Sciences, El-Oued University, El Oued 39000, Algeria. E-mail: dersamebio@gmail.com. Phone: +213-669-56-62-66

Received: 16 May 2020 / Accepted: 5 August 2020

Abstract

Introduction: The aim of the present study was to evaluate the effect of purslane (*P. oleracea*) and Aquilaria malaccensis (*A. malaccensis*) methanol extracts against high-fructose and high-fat diet induced insulin-resistance, lipid peroxidation and tissues dysfunction in rats. **Material and Method:** Females albino Wistar rats were divided into six groups (n=5) as control, Insulin resistance rats (IR), IR+Po, IR+Am, IR+Po+Am and IR+Met groups. Insulin resistance (IR) in rats was induced by diet with a high fructose (60% fructose) and a high fat diet (60% kcal fat) for 70 days. *P. oleracea* (Po) and *A. malaccensis* (Am) methanol extracts and metformin drugs were supplemented orally (400 mg/kg bw, 200 mg/kg bw, and 300 mg/kg bw), respectively, for four weeks. Methanol extracts of plants were prepared and phytochemicals were analyzed by standard methods. Blood glucose level, lipids profile, lipid peroxidation and some biochemical parameters were assessed. **Results:** Obtained results revealed that IR induction caused a significant increase in blood glucose, plasma and tissues lipids profile, urea and creatinine concentrations, GOT, GPT, and ALP activities compared to the control group while total protein concentration was significantly lower. Additionally, there was a significant increase of MDA level in IR group than those of the control. Methanol extracts of *P. oleracea* and *A. malaccensis* treatment ensured a partial correction of the previous parameters. **Conclusions:** The results of the present investigation indicated that *P. oleracea* and *A. malaccensis* possesses the ability to control the lipid peroxidation and biochemical disruption associated with insulin resistance.

Keywords: *A. malaccensis,* High Fructose Diet, Insulin-resistance, *P. oleracea,* Rats.

Introduction

Insulin resistance is a common feature of metabolic diseases and is the leading cause of type 2 diabetes (T2D). The prevalence of T2D worldwide is extremely high (8.8% of the world population) and is growing rapidly. In 2015, T2D killed about 5 million people [1]. The pre-diabetic state preceding T2D is characterized by obesity, insulin resistance and as it has been demonstrated during in the last decade, chronic low-grade inflammation [2], is manifested by a progressive increase in insulinemia due to a lack of insulin signaling in target metabolic organs, such as the liver, muscle and adipose tissue [3]. Insulin resistance can be broadly defined as insulin-reduced cellular reactivity, characterized by higher insulin levels needed to maintain peripheral glucose levels [4]. Nutrition characterizes a lifestyle element that can be measured, and that can directly impact health; consequently, defensive nutrition and weight control should develop a main focus of consumers and prepared-food providers [5]. Food imbalance is a major risk factor for many pathologies such as cardiovascular diseases, diabetes and obesity [6]. Oxidative stress is an important pathogenic mechanism of

© 2020 The Authors. Romanian Journal of Diabetes, Nutrition and Metabolic Diseases published by Sanatatea Press Group on behalf of the Romanian Society of Diabetes Nutrition and Metabolic Diseases. This is an open access article under the terms of the Creative Commons Attribution License (CC-BY-NC-ND 3.0).

the metabolic syndrome associated with insulin resistance and plays a crucial role in the pathogenesis of various diseases [7]. Current treatment, particularly with metformin, does not adequately address the issue of insulin resistance. Therefore, it is necessary to search for new agents with better efficacy and minimal side effects [8]. Since ancient times, herbs and plants have been used as medicines for many diseases, they are still considered as the basis of a system of traditional medicine in different cultures [9]. Among these medicinal plants is Portulaca oleracea which is a traditional vegetable is used by indigenous and tribal peoples in many countries. It is known to contain many biologically active compounds and is also reported as a source of many nutritional supplements [10]. On the other hand, *Aquilaria malaccensis* is a species of tropical plants of the family Thymelaeaceae [11]. It is one of the main sources of agar wood, which provides clues about their pharmacological properties. Indeed, agar wood contains several bioactive compounds that now elegantly support their use in traditional medicine [12]. Thus, the aim of this study is to evaluate the modulatory effect of the methanol extracts of *Portulaca oleracea* leaves and the trunk bark of *Aquilaria malaccensis* against high fructose and high fat diet induced insulin resistance metabolic disturbance and lipid peroxidation in rats.

Materials and Methods

Drugs and chemicals

Metformin was supplied in the form of tablets (Metformine Zentiva tablets, SAIDAL Group, Algiers, Algeria) and is given as a suspension in distilled water. Fructose was supplied as powder (Biomax, Specialized Food Industry, Algiers, Algeria). All other chemicals used in this study are of fine analytical grade.

Plant material

The plants used in this study are the bark trunk of Aquilaria malaccensis (A. *malaccensis*) which were purchased from the local market and the leaves of Portulaca oleracea (P. *oleracea*) were harvested in the region of El-Oued "Guemar" in September 2016. The identity of plant was confirmed by a botanist at the herbarium in the Department of biology, the University of El Oued. The bark of A. *malaccensis* and leaves of P. *oleracea* were cleaned, dried in shade, powdered and then stored in air tight container until the beginning of the experiment.

Preparation of methanol extracts

The crude samples (each 3.75 g) were extracted by shaking with 25 ml of aqueous methanol (70:30) at 25 ° C for 48 h and filtered through filter paper. The residues were re-extracted with an additional 18.75 ml of aqueous methanol, as described above, for 3 hours. After extraction, the solvents were removed under low temperature (40 ° C) using a rotary evaporator.

Chemical characterization

Phytochemical Screening

Phytochemical tests were performed on extracts prepared from the plant by qualitative characterization techniques using standard screening test and phytochemical procedures.

Estimation of Total Phenol

The polyphenols were determined by the Folin-Ciocalteu method. This method, initially described by Slinkard and Singleton [13], makes it possible to know the total polyphenolic content of a given sample. The sample of the methanol extract of A. *malaccensis* or P. *oleracea* (0.5 ml) and 2 ml of sodium carbonate (75 g/l) were added to 2.5 ml of 10% (v/v) Folin- Ciocalteau with gallic acid as standard. After 30 min of reaction at room temperature, the absorbance was measured at 765 nm. The tests were carried out three times in order to ensure the reproducibility of the results. The total phenolic content was expressed in mg equivalent of gallic acid per gram of sample.

https://doi.org/10.46389/rjd-2020-1052
© 2020 The Authors

Estimation of Total Flavonoids

Determination of the total flavonoid content of the methanol extract of A. *malaccensis* and P. *oleracea* was carried out by the method described by Lin and Tang [14]. 0.5 ml of a 2% $AlCl_3$ ethanol solution was added to 0.5 ml of sample or standard. After 1 hour at room temperature, the absorbance was measured at 420 nm. Quercetin was used as a standard for plotting the calibration curve. The tests were carried out three times in order to ensure the reproducibility of the results. The results were expressed in milligram equivalent to Quercetin per gram of sample.

In-vivo study

Animals and treatment

Adult female albino rats (aged between 8–10 weeks), weighing 200–270 g, were taken from the animal house of Pasteur institute, Algeria. They were placed in six groups of five rats each and kept in animal house of Molecular and cellular biology Department, University of El Oued, Algeria. Animals were adapted for two weeks under the same laboratory conditions with a relative humidity 64.5% and room temperature of 25 ± 2 C°. After acclimation period, the rats were fed with either a high fructose diet, which is composed of 60% fructose (substituted for carbohydrates in the standard laboratory diet) and a high fat diet (60% kcal fat, 20% kcal carbohydrates, 20% kcal protein) (HFFD), or a normal chow diet (10% kcal fat, 70% kcal carbohydrates, 20% kcal protein) for 70 days. Mice were kept on a photoperiod (12 hr light/12 hr dark) and were fed ad libitum. After 60 days of feeding, the HFFD fed rats showed obvious phenotypes of insulin resistance (IR) compared to the normal diet control group. We then randomly divided them into six groups each containing five rats.

Group 1 (control group): animals were given normal diet served as control.

Group 2 (IR): insulin resistance rats were given HFFD diet.

Group 3 (IR+Po): Insulin resistance rats were given normal diet plus methanol extracts of P. *oleracea* (400 mg kg^{-1} d^{-1}) administered orally.

Group 4 (IR+Am): Insulin resistance rats were given HFFD diet plus methanol extracts of A. *malaccensis* (200 mg. kg^{-1} .d^{-1}) administered orally.

Group 5 (IR+Po+Am): Insulin resistance rats were given HFFD diet plus both methanol extracts of P. *oleracea* and A. *malaccensis* administered orally.

Group 6 (IR+Met): Insulin resistance rats were given HFFD diet plus Metformine (300 mg.kg^{-1}. d^{-1}) administered orally.

Animals were maintained in the appropriate experimental treatments for 30 days. Body weight was recorded regularly. The experimental procedures were carried out according to the National Institute of Health Guide-lines for Animal Care and approved by the Ethics Committee of our Institution.

Blood collection and preparation of tissue samples

At the end of treatment, rats were fasted for 16 hours, anaesthetized with chloroform by inhalation, then rats were decapitated and blood samples were transferred into ice cold centrifuge tubes. The serum was prepared by centrifugation, for 10 min at 3000 revolutions/min and utilized for triglyceride, total cholesterol, HDL, urea, protein, creatinine concentrations and GOT, GPT, and ALP activities assays. The blood glucose was measured by glucometer. Absolute liver and kidney weight was determined while liver, kidney, and pancreas were rapidly excised, weighed and stored at – 20°C for lipid peroxidation analysis.

Measurement of biochemical parameters

The activities of glutamate-oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), alkaline phosphatase (ALP) were determined using commercial kits from Spinreact (Girona, Spain), triglyceride (TG), total cholesterol (TC), high density lipoprotein (HDL), total proteins, urea, and

© 2020 The Authors

creatinine concentrations were also measured using commercial kits obtained from Spinreact (Girona, Spain).

Lipid peroxidation measurement

Preparation of homogenates

1g of liver, kidney or pancreas was homogenized in 9 ml of buffer solution (phosphate buffer saline, pH=7.4). Homogenates were centrifuged at 9000xg for 15 min at 4°C, and the obtained supernatant was used for the determination of MDA parameter.

Estimation of Malondialdehyde (MDA) levels

Lipid peroxidation process was determined in supernatant of homogenate liver, kidney and pancreas tissues by the thiobarbituric acid (TBA) method which estimates the malondialdehyde formation (MDA) according to Sastre et al. (2000) [15]. Absorbance of TBA-MDA complex was determined at 530 nm and the level of hepatic MDA was expressed as µmoL/mg protein.

Statistical Analysis

The present data was reported as Mean ± SEM. The significance of differences was calculated by using 1-way analysis of variance followed by the Student t-test to compare means among the groups. Differences were considered statically significant at $p<0.05$.

Results

Phytochemical screening, Phenolic and Flavonoid Compounds

The results of phytochemical tests of various extracts which are presented in table 1 clearly show that the extracts of P. *oleracea* and A. *malaccensis* are rich in secondary metabolites including flavonoids tannins and saponins. From the results of quantitative analysis shown in Table 1, it was noted that the content of phenolic compound in the methanol extract of P. *oleracea* and A. *malaccensis* was very important which has 6.22 mg and 2.60 mg GAE per g of dry extract of each plant respectively. Similarly, Table 1 shows that the flavonoid content in A. *malaccensis* and P. *oleracea* extract is important, which has 0.255 and 0.528 mg QE per g of the dry extract of each plant, respectively.

Initial body weight, body weight gain and relative weight of liver and kidney

Our results (Fig 1) show that the induction of insulin resistance causes a significant increase ($p<0.01$) of weight gain in rats compared to the control group. On the other hand, a significant decrease in weight gain is noticed in IR +Po

Table 1: Qualitative and quantitative analysis of phytochemical composition of methanol extract of P. *oleracea* and A. *malaccensis* (+ presence)

	Compounds	P. *oleracea*	A. *malaccensis*
Qualitative Analysis	Flavonoids	+	+
	Tannins	+	+
	Alkaloids	+	+
	Saponines	+	+
	Glycosides	+	+
Quantitative Analysis	Total phenolic (mg GAE/g extract)	6.226±0.189	2.609±0.094
	Flavonoids (mg QE/g extract)	0.255±0.011	0.528±0.007

https://doi.org/10.46389/rjd-2020-1052
© 2020 The Authors

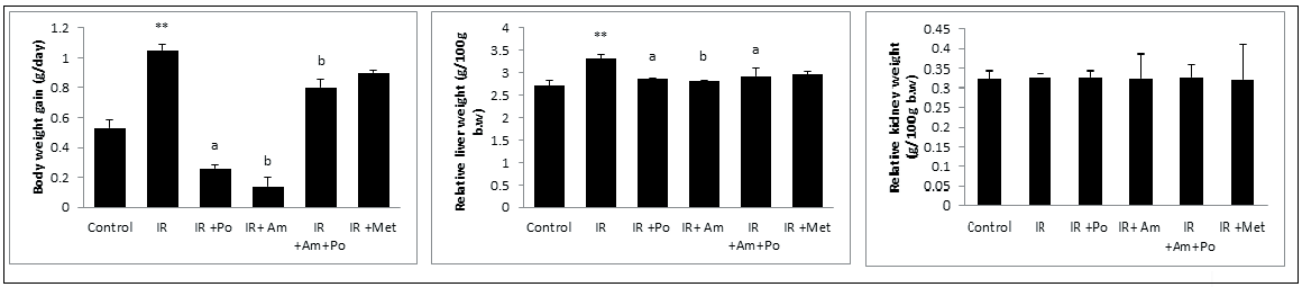


Figure 1: Mean body weight gain and relative liver and kidney weight of control and experimental rats. **p<0.01: significantly different from control group. [a] p<0.05, [b] p<0.01: significantly different from IR group.

(p<0.05), IR+ Am (p<0.001), IR +Am+ Po (p<0.01) and IR +Met (p<0.01) groups compared to the IR group (Table 3). The results obtained show a significant increase in the relative liver weight (p<0.01) in the IR group compared to the control group, but no change in the relative kidney weight. Treatment with *P. oleracea* and *A. malaccensis* extracts or both completely improves this anomaly compared to the IR group.

Blood biochemical values

As seen from Table 2, blood glucose level and serum lipid concentration was significantly altered which increased the level of glucose (p<0.01), cholesterol, triglycerides (p<0.001), low density lipoprotein (LDL) (p<0.01), very low density lipoprotein (VLDL) (p<0.001) liver triglycerides (p<0.01), liver cholesterol (p< 0.001), adipocytes triglycerides (p<0.001) and Adipocytes cholesterol (p<0.05) at the end of the treatment period in IR animals as compared to normal animals. Moreover, the results obtained show that there is a significant improvement in the blood glucose level, serum and tissues triglyceride, cholesterol, VLDL and LDL concentrations in the rats treated with extract of *P. oleracea* (Po), *A. malaccensis* (Am), Po + Am and Metformin (Met) compared to IR group while only treatment significantly increases the HDL level compared to the IR group.

Liver and renal function markers

As indicated in Table 3, insulin resistance induced a renal dysfunction with a significant (p<0.05) increase in urea and creatinine

Table 2: Mean blood glucose and lipid profile in serum and tissues of control and experimental rats.

Groups	Control	IR	IR +Po	IR+ Am	IR +Am+ Po	IR +Met
Blood Glucose (mg/dl)	101±2	182±4***	109±9.1^{b}	109±8.2^{b}	115±1.3^{*b}	117±1.2^{**b}
Serum TG (mg/dl)	83.5±6.4	177.2±4.5**	104±6. 49^{c}	64.25±8.28^{c}	73.50±5.42^{c}	71.50±5.32^{c}
Serum TC (mg/dl)	54.2±4.3	83.5±2.2**	51.7±2.2^{b}	52.0±1.2^{b}	54.7±1.9^{b}	53.0±5.3^{b}
Serum HDL (mg/dl)	17.6±0.8	15.1± 1*	17.0±0.2^{b}	17.4±0.5^{b}	16.4±0.9*	16.7±0.7
Serum LDL (mg/dl)	16.2±1.6	29.4±1.5***	15.6±2.8^{c}	19.2±2.1^{c}	19.5±0.03.5^{c}	20.4±2.2^{*c}
Serum VLDL (mg/dl)	16.7±1.3	35.44±0.9**	20.8±1.29^{c}	12.85±1.65^{c}	14.7±1.08^{c}	14.3±1.06^{c}
Liver TG (mg/g tissue)	5.13±0.27	9.18±0.27**	6.03±0.36	5.13±0.09^{c}	5.76±0.27^{b}	6.21±0.27^{*b}
Liver CHL (mg/g tissue)	5.22±0.09	7.74±0.26***	5.76±0.18^{a}	4.95±0.25^{b}	5.85±0.17^{*b}	6.39±0.18^{*a}
Adipocytes TG (mg/g tissue)	10±0.8	50.8±2.9***	38.6±3.5^{***a}	35.46±1.9^{***b}	37.3±1.2^{***a}	37.5±0.6^{***a}
Adipocytes CHL (mg/g tissue)	1.62±0.09	2. 88±0.16*	2.16±0.054^{a}	1.80±0.09^{b}	2.43±0.18*	2.25±0.09*

Data is expressed as Mean ± SEM, (n=5 animals/group).
*p<0.05, **p<0.01, ***p<0.001: significantly different from Normal group.
[a] p<0.05, [b] p<0.01, [c] p<0.001: significantly different from IR group

© 2020 The Authors

concentration and a significant decrease (p<0, 01) of total protein concentration in rats of IR group compared to controls. On the other hand, treatment with the methanol extract of *P. oleracea* (Po), *A. malaccensis* (Am) or Po+Am corrects these disturbances compared to the untreated IR rats. For liver function markers, the results showed a significant (p<0.05) increase in transaminases activities (GOT and GPT) and alkaline phosphatase in IR group compared to control. In contrast, a significant decrease (p<0.05) of GOT and GPT activities was reported under the effect of treatment with extract of *P. oleracea* (Po) or *A. malaccensis* (Am) and a decrease in ALP and GOT activities in the group treated with Po + Am compared to untreated IR rats. Regarding treatment with metformin, results didn't notice any significant improvement in liver function in comparison with the IR group.

Lipid peroxidation marker

The obtained results (Fig. 2) showed a significant increase (p<0.05) of lipid peroxidation in the liver, kidneys and pancreas in insulin resistance (IR) group. While treatment with *P. oleracea* (Po), *A. malaccensis* (Am) or Por + Aq leads to a significant reduction (p<0.05) in hepatic, renal and pancreatic MDA levels compared to the IR group. Finally, there was only a significant decrease in pancreatic MDA concentration in the metformin-treated rats compared to the IR group.

Discussion

The results of the phytochemical analysis revealed that the methanol extract of dry leaves of *Portulaca oleracea* and the trunk bark of *Aquilaria malaccensis* contains several bioactive compounds, including polyphenols, saponins, tannins, alkaloids, and flavonoids. Dietary phytochemicals can act on one or more molecular targets that relieve multiple pathological processes, including oxidative damage, epigenetic alterations, chronic inflammation, active stimulators, inhibitors and growth terminators and prevention of various diseases associated with oxidative stress [16]. In our study, results showed

Table 3: Mean serum kidney function markers and enzymes activities in control and experimental rats.

Groups	Control	IR	IR +Po	IR+ Am	IR +Am+Po	IR +Met
Serum creatinine (mg/l)	8.3±0.15	10.40±0.47*	8.42±0.35[a]	8.15±0.22[b]	7.950±0.290[a]	7.900±0.10[a]
Serum urea (g/l)	0.58±0.03	0.87±0.04*	0.66±0.02*[a]	0.62±0.03[b]	0.715±0.04*[a]	0.64±0.05[a]
Serum protein (mg/l)	46.21±2.18	32.83±0.51**	38.78±1.11[b]	39.20±1.46[b]	34.02±1.06*	36.31±0.52*[a]
Serum ALP (U/l)	38.30±1.30	43.86±1.49*	38.13±2.36**	37.74±1.84	38.96±1.08	39.32±1.57
Serum GOT (U/l)	132.7±1.32	143.89±1.21*	134.18±2.71[a]	131.96±1.24[a]	133.51±3.52[a]	130.34±4.91[a]
Serum GPT (U/l)	36.42±1.4	53.12±0.9**	33.06±2.76[b]	34.63±1.44[b]	31.16±3.05[b]	33.30±6.65

Data is expressed as Mean ± SEM, (n=5 animals/group).
*p<0.05, **p<0.01, ***p<0.001: significantly different from Normal group.
[a]p<0.05, [b]p<0.01, [c]p<0.001: significantly different from IR group.

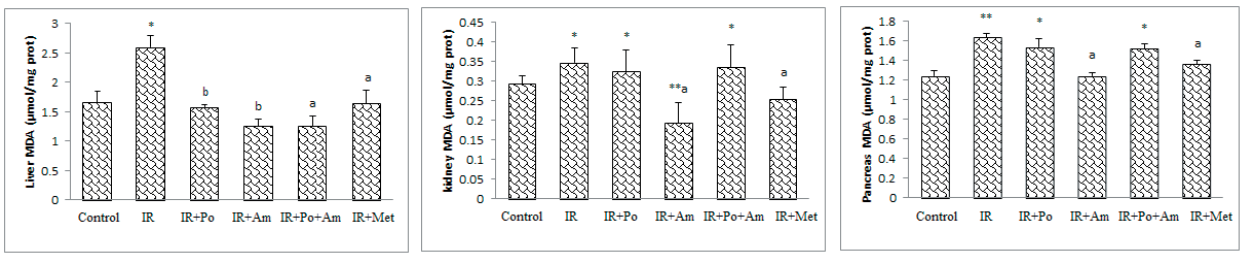


Figure 2: Mean MDA level in liver, kidney and pancreas tissues of control and experimental rats. *p<0.05, **p<0.01: significantly different from control group. [a] p<0.05, [b] p<0.01: significantly different from IR group.

https://doi.org/10.46389/rjd-2020-1052
© 2020 The Authors

that in rats receiving a high fat diet with high fructose causes a significant increase in weight gain and relative liver and kidney weight resulting in an obese phenotype. More consumption of high energy content nutriments such as fructose and HFD leads to rise in the fat mass and fat cell expansion [hypertrophy) without changing food intake, producing the specific pathology of obesity [17]. Also high proportion of lipids in food can increase palatability and cause hyperphagia of animals leading to rapid weight gain [18]. Treatment of insulin-resistant rats with the methanol extract of P. *oleracea* and/ A. *malaccensis* induces a decrease in body weight gain. The anti-obesity properties of plants can be exerted according to different modes of action: by direct effect on food intake by suppressing appetite and inducing the feeling of satiety, a reduction of lipid absorption, a reduction of energy consumption, increased energy expenditure, decreased pre-adipocyte differentiation and proliferation, decreased energy intake from the gastrointestinal tract [19]. Our results show a disruption of lipid and carbohydrate metabolism under the effect of HFFD regime. The Cafeteria diet also leads to excessive development of adipose tissue which could be due to a decrease in the ability to oxidize dietary lipids and enlargement of adipocytes (hypertrophy). In addition, fat accumulation is regulated by the lipolysis cycle, lipogenesis [20]. This explains the high level of triglycerides and cholesterols in adipose tissue in diet-fed rats (HG + F] compared with standard diet rats, this is consistent with the BOCARSLY et al. (2010) study which shows high fructose content causes characteristics of obesity in rats, increased body weight and body fat [21]. In rodents, a high-fructose diet leads to the development of obesity insulin resistance, suggesting that fructose is the main mediator of glucose intolerance and insulin resistance [22]. Fructose has a less inducing mechanism of satiety than glucose / sucrose, and will also decrease the sensitivity of the liver and peripheral tissues to insulin which is explained as hyperglycemia [23]. Treatment with A. *malaccensis* induces a reduction in hyperglycemia and dyslipidemia, the anti-hyperglycemic agent and glucose uptake enhancement activities of methanol extracts of A. *malaccensis* are similar to those of insulin [24]. In addition, A. *malaccensis* is rich in flavonoids which is able to reduce the increase in blood glucose levels and increase glucose uptake in rat muscle more effectively than insulin [25]. Our results also showed that treatment with P. *oleracea* exerts a hypoglycemic, hypolipedemic effect and significantly reduces weight gain. The anti-hyperglycemic activity of P. *oleracea* can be attributed to the presence of polyphenols reported as a major role in the reduction of diabetes that helps regulate plasma glucose levels and hepatic glucose metabolism which could inhibit digestive enzymes such as salivary amylase, intestinal sucrose and α-glucosidase. This reduced the action of digestibility and promoted the regeneration of pancreatic β cells and increase the muscle glucose transporter [26]. The results show hepatic and renal dysfunction and increase in lipid peroxidation induced by fructose diet. Liver tissue lesions due to hyperlipidemia impair their transport function and membrane permeability, leading to enzyme leakage from cells, therefore, marked release of GOT and GPT in the circulation indicates severe damage to hepatic tissue membranes [27]. Indeed, elevation of creatinine and urea and decrease in protein may explain the diabetic nephropathy which is the main determinant of morbidity and mortality in patients with diabetes. Chronic hyperglycemia is a major initiator of disruption of renal function [28]. High fructose and HFD has been identified to increase the tissues mitochondrial reactive oxygen species (ROS) production. Excessive ROS production under insulin resistance/hyperglycemic conditions attacks local cell organelles, including membrane lipids, resulting in lipid peroxidation [29]. In contrast, treatment with P. *oleracea* and/ or A. *malaccensis* shows a significant decrease in ASAT, ALAT and PAL activities and module urea and creatinine concentrations compared to insulin-resistance rats. This protective action may be due to the improvement of fat accumulation in the liver and a rapid restoration of insulin sensitivity in the liver, at least at an early stage of hepatic insulin resistance [30]. In fact, the hypoglycaemic effects of two plants may be associated with nephroprotection, flavonoids

© 2020 The Authors

having a protective effect on renal dysfunction in rats which were fed a high fructose diet, by modulating the pathological pathways induced insulin resistance.

Conclusion

In conclusion, this study clearly shows that the Methanol extract of bark A. *malaccensis* and leaves of P. *oleracea* possess the ability to control the biochemical disruption associated with insulin resistance, antioxidant action and protective activity on liver, kidney and pancreatic cells, which in turn will improve the energy metabolism.

Acknowledgements

This work was supported by the research project D01N01UN390120190001 funded by the ministry of higher education, Algeria and by Directorate general for Scientific Research and Technological Development.

Conflict of interest

The authors declare no conflict of interest.

References

1. Bruna BB, Beatriz AG, Marcelo AM. Shortcuts to a functional adipose tissue: The role of small non-coding RNAs. *Redox Biol.* 12: 82–102, 2017.
2. Hameed I, Masoodi SR, Mir SA, Nabi M, Ghazanfar K, Ganai BA. Type 2 diabetes mellitus: From a metabolic disorder to an inflammatory condition. *World Journal of Diabetes*. 6(4):598–612, 2015.
3. Scherer T, Lindtner C, O'Hare J, et al. Insulin Regulates Hepatic Triglyceride Secretion and Lipid Content via Signaling in the Brain. *Diabetes*. 65(6):1511–1520, 2016.
4. Cheke LG, Bonnici HM, Clayton NS, Simons JS. Obesity and insulin resistance are associated with reduced activity in core memory regions of the brain. *Neuropsychologia*. 96:137–149, 2017.
5. Basciano H, Federico L and Adeli K. Fructose, insulin resistance, and metabolic lipidemia. *Nutrition & Metabolism*. 2:5, 2005.
6. Martín-Timón I, Sevillano-Collantes C, Segura-Galindo A, del Cañizo-Gómez FJ. Type 2 diabetes and cardiovascular disease: Have all risk factors the same strength? *World Journal of Diabetes*. 5(4):444–470, 2014.
7. Derouiche S, Degachi O, Gharbi K. Phytochemistry analysis and modulatory activity of Portulacae oleracea and Aquilaria malaccensis extracts against High-fructose and high-fat diet induced immune cells alteration and heart lipid peroxidation in Rats. *International Research Journal of Biological Sciences*. 8(4): 6–11, 2019.
8. Bacha F, Klinepeter Bartz S. Insulin resistance, role of metformin and other non-insulin therapies in pediatric type 1 diabetes. *Pediatr Diabetes*. 17(8):545–558, 2016.
9. Rakotoarivelo NH, Rakotoarivony F, Ramarosandratana AV, et al. Medicinal plants used to treat the most frequent diseases encountered in Ambalabe rural community, Eastern Madagascar. *Journal of Ethnobiology and Ethnomedicine*. 11:68, 2015.
10. Mubashir H M, Bahar A, Showkat RM, Bilal AZ, Nahida T. Portulaca oleracea L. A Review. *Journal of Pharmacy Research*. 4(9): 3044–3048, 2011.
11. Dolikajyoti S, Hemen S, Sujata H, Islam NF, Prasad MNV. "Agro-Ecosystem Diversity in Petroleum and Natural Gas Explored Sites in Assam State, North-Eastern India: Socio-Economic Perspectives". *Sustainable Agriculture Reviews*. 27: 37–60, 2018.
12. Hashim YZ, Kerr PG, Abbas P, Mohd Salleh H. Aquilaria spp. (agarwood) as source of health beneficial compounds: A review of traditional use, phytochemistry and pharmacology. *J Ethnopharmacol*. 189: 331–60, 2016.
13. Slinkard K, Singleton VL. Total Phenol Analysis: Automation and Comparison with Manual Methods. *Am J Enology Vitic*. 28: 49–55, 1977.
14. Lin JY, Tang CY. Determination of total phenolic and flavonoid contents in selected fruits and vegetables, as well as their stimulatory effect on mouse splenocyte proliferation. *Food Chem*. 101:140–7, 2007.
15. Sastre J, Pallardó FV, García de la Asunción J, Viña J. Mitochondria, oxidative stress and aging. *Free Radic Res*. 32(3):189–98, 2000.
16. Kaouachi A, and Derouiche S. Phytochemical analysis, DPPH antioxidant activity and Acute toxicity of bark aqueous extracts of Pinus halepensis. *Res. J. Chem Env Sci*. 6(3): 86–91, 2018.
17. Fontaine KR, Redden DT, Wang CA, Westfall O, Allison DB. Years of life lost due to obesity. *The Journal of the American Medical Association*. 289(2): 187–193, 2003.
18. Oliva L, Aranda T, Caviola G, Fernández-Bernal A, Alemany M, Fernández-López JA, Remesar X. In rats fed high-energy diets, taste, rather than fat content, is the key factor increasing food intake: a comparison of a cafeteria and a lipid-supplemented standard diet. Peer J. 2017; 5:e3697 https://doi.org/10.7717/peerj.3697.
19. Jia Zheng, Sheng Zheng, Qianyun Feng, Qian Zhang, Xinhua Xiao. Dietary capsaicin and its anti-obesity potency: from mechanism to clinical implications. *Bioscience Reports*. 37 (3), 2017. BSR20170286; doi: 10.1042/BSR20170286.
20. Derouiche S, Abbas K, Djermoune M. Polysaccharides and ascorbic acid content and the effect of aqueous extract of portulaca oleracea in high-fat diet-induced obesity, dyslipidemia and liver damage in albino wistar rats. *Algerian journal of arid environment*. 7(2): 16–26, 2017.
21. Bocarsly ME, Powell ES, Avena NM, Hoebel BG. High-fructose corn syrup causes characteristics of obesity in rats: increased body weight, body fat and triglyceride levels. *Pharmacology, biochemistry, and behavior*. 97(1):101–106, 2010.

 https://doi.org/10.46389/rjd-2020-1052 © 2020 The Authors

22. Thresher JS, Podolin DA, Wei Y, Mazzeo RS, Pagliassotti MJ. Comparison of the effects of sucrose and fructose on insulin action and glucose tolerance. *Am J Physiol Regul Integr Comp Physiol*. 279(4):R1334–40, 2000.
23. Rendeiro C, Masnik AM, Mun JG, et al. Fructose decreases physical activity and increases body fat without affecting hippocampal neurogenesis and learning relative to an isocaloric glucose diet. *Scientific Reports*. 5:9589, 2015. doi:10.1038/srep09589.
24. Ratree P, Patchareewan P, Chantana A. Antihyperglycemic activity of agarwood leaf extracts in STZ-induced diabetic. rats and glucose uptake enhancement activity in rat adipocytes. *Songklanakarin J Sci Technol*. 33(4): 405–410, 2011.
25. Vinayagam R, Xu B. Antidiabetic properties of dietary flavonoids: a cellular mechanism review. *Nutrition & metabolism*. 12:60, 2015. DOI:10.1186/s12986-015-0057-7.
26. Samah AliEl-Newary. The hypolipidemic effect of Portulaca oleracea L. stem on hyperlipidemic Wister Albino rats. *Annals of Agricultural Sciences*. 61(1): 111–124, 2016.
27. Ahn MR et al. Antioxidant activity and constituents of propolis collected in various areas of China. *Food Chem*. 101: 1383–1392, 2007.
28. Das Evcimen N, King GL. The role of protein kinase C activation and the vascular complications of diabetes. *Pharmacol Res*. 55(6): 498–510, 2007.
29. Derouiche S, Azzi M, Hamida A. Effect of extracts aqueous of phragmites australis on carbohydrate metabolism, some enzyme activities and pancreatic islet tissue in alloxan induced diabetic rats. *Int. J. Pharm. Pharm. Sci*. 9(6): 54–58, 2017.
30. Hwang KA, Hwang YJ, Kim GR, Choe JS. Extracts from Aralia elata (Miq) Seem alleviate hepatosteatosis via improving hepatic insulin sensitivity. *BMC Complement Altern Med*. 15:347, 2015. doi: 10.1186/s12906-015-0871-5.

© 2020 The Authors

https://doi.org/10.46389/rjd-2020-1053
www.rjdnmd.org

Rom J Diabetes Nutr Metab Dis
2020; volume 27, issue 4, pages 366-371

Original Research

Serum levels of 8-hydroxy 2-deoxyguuanosine as a marker of DNA damage in healthy obese individuals

Abdelmarouf Hassan Mohieldein

Associate Professor of Medical Biochemistry, Department of Medical Laboratories, College of Applied Medical Sciences, Qassim University, Buraidah, Saudi Arabia

*Correspondence to: Abdelmarouf Hassan Mohieldein, Department of Medical Laboratories, College of Applied Medical Sciences, Qassim University. P.O. Box 6699 Buraidah 51452, Qassim, Kingdom of Saudi Arabia. E-mail: mabdelmarouf@hotmail.com, Phone: +966-556876251, Fax: 00966 6 3801628

Received: 20 May 2020 / Accepted: 8 August 2020

Abstract

Background and Aims: Obesity is a serious and growing healthcare concern worldwide. It is associated with mortality and co-morbidity. We aimed to determine serum concentrations of 8-hydroxy-2`-deoxyguanosine and their association with body-mass index in healthy individuals. **Material and Method:** Fifty-nine healthy individuals were recruited from public places in this cross sectional study. Participants were divided into: obese group and non-obese group. Blood collected; serum and plasma were prepared. Glucose and glycated hemoglobin were assayed by standard methods using commercial kits. 8-hydroxy-2` -deoxyguanosine was determined by enzyme-linked immunosorbent assay. Data were analyzed using software Statistical Packages for the Social Sciences. **Results:** Data showed that median (interquartile levels) of serum 8-hydroxy-2`-deoxyguanosine was significantly higher in the obese subjects in comparison with controls. Moreover, a positive correlation was documented between level of serum 8-hydroxy-2`-deoxyguanosine and body-mass index in study participants. **Conclusions:** The study findings suggest that weight loss for obese individuals could reduce DNA damage and oxidative stress which underlie the pathogenesis of obesity-associated metabolic disorders.

Keywords: Healthy individuals, Obesity, Oxidative DNA damage, Oxidative stress, Reactive oxygen species.

Background and Aims

Obesity is a serious and growing healthcare concern worldwide [1, 2]. The prevalence of obesity was 55% in Europe, 46% in Eastern Mediterranean, 26.9% in Africa and 35.7% in USA [3, 4]. Obesity is associated with co-morbidities including: cardiovascular, diabetes mellitus, cancer, hypertension and metabolic disorders [5]. Moreover, Flegal KM et al., [6] reported that high level of obesity was associated with increased relative risks of mortality, which is partially attributed to increased risk for chronic diseases especially when obesity starts at an early age [7]. One consequence of obesity is the insulin metabolic dysregulation for free fatty acids and glucose [8].

Cellular respiration and multiple enzyme systems such as P450 monoxygenase system can generate reactive radicals [9]. Although the human body has defense mechanisms against these reactive radicals, sometimes they may not function optimally and result in oxidative damage, as is seen in obesity and its associated complications [10–12]. In obese subjects, oxygen-derived radicals—such as reactive oxygen species (ROS)—and decreased antioxidants disrupt the redox system [13, 14]. ROS, oxygen-containing molecules with or without unpaired electrons, are highly reactive in tissues [15]. They oxidize macromolecules like lipids, proteins and DNA [11]. Thus, oxidative damage markers can be identified: malondialdehyde for lipid peroxidation; advanced glycosylated end

© 2020 The Authors. Romanian Journal of Diabetes, Nutrition and Metabolic Diseases published by Sanatatea Press Group on behalf of the Romanian Society of Diabetes Nutrition and Metabolic Diseases. This is an open access article under the terms of the Creative Commons Attribution License (CC-BY-NC-ND 3.0).

products for protein oxidative modification; and 8-hydroxy-2'-deoxyguoanosine (8-OHdG) for oxidatively modified products of DNA [16]. Different forms of DNA damage in human include: single strand breaks, double strand breaks, mis-pairing, and base modification [17]. An example of the latter is 8-OHdG, which is an oxidized purine base in DNA [18]. It is a sensitive biomarker for oxidative DNA damage [19]. Researchers documented increased concentration of 8-OHdG in different human specimens in many diseases including diabetes, cardiovascular, cancer etc [20].

Despite the huge literature of studying 8-OHdG in obesity associated co-morbidities, limited number of studies explored 8-OHdG in healthy obese subjects. Herein, we hypothesized that obese healthy individuals have increased serum 8-OHdG than non-obese subjects. Therefore, the study aims to determine serum 8-OHdG in healthy obese individuals in comparison with non-obese healthy individuals. We will also examine the relationship between 8-OHdG and body mass index (BMI) among healthy obese individuals.

Materials and Methods

Study Design and participants

In this cross sectional study, fifty-nine healthy individuals were recruited from public places. Based on World Health Organization criteria of obesity [21], participants were further divided into two group: obese group and non-obese group. The study excluded participants who reported they had chronic diseases such as: diabetes mellitus, hypertension, or cardiovascular disease. In addition, random blood glucose levels <11.1 mmol/l and glycated hemoglobin (HbA1c) <5.5% were considered as confirmatory criteria to enroll participants in the study.

Laboratory, anthropometric and clinical data collection

Body weight and height were measured for each participant. BMI was calculated as body weight (kilogram) divided by height (meter) squared. Participants with BMI ≥30 Kg/m^2 were classified as obese while those with BMI <30 Kg/m^2 were considered as non-obese.

After verbal consent, each participant agreed to give blood samples in plain and heparinized vacutainers. Blood samples in vacutainers was allowed to clot, then centrifuged at 3000 rpm for 15 minutes to get serum. While blood in heparinized vacutainers was spun at 3000 rpm for 10 minutes to get plasma.

We used commercial kit manufactured by Human Diagnostics, Wiesbaden, Germany for measurement of blood glucose. The principle is based on Glucose oxidase-glucose peroxidase method. Kits based on immunoturbidmetric method were used to assay HbA1c. They were purchased from Vital Diagnostic, Italy. The serum levels of 8-OHdG were determined by using a competitive enzyme-linked immunosorbent assay kit which was obtained from the Northwest Life Science Specialties, U.S.A

Statistical Analysis

IBM SPSS statistics (version 23) software was used to analyze data. Variables were compared with the normal distribution using the skewness/kurtosis measures. Nonparametric methods were applied when data were not normally distributed. Descriptive statistics (mean ± standard deviation or number (percentage) were used for continuous or categorical variable respectively. In addition, level of 8-OHdG was expressed as median (interquartile) and mean ± standard deviation.

Differences between the two groups (obese vs. non-obese) were compared using chi square, unpaired t-test or the Mann-Whitney U-test where appropriate. Spearman correlation test was performed to examine the relationship BMI and 8-OHdG. We considered results to be significant at $p<0.05$.

Ethical Consideration

The study was conducted in accordance with the Declaration of Helsinki. After thorough explanation of the goals of the study, each

© 2020 The Authors

participant gave verbal consent. Participation was voluntary and confidentiality of all participants was maintained as no names were requested.

Results

Characteristics & Laboratory investigations of study participants

Data show no significant difference in the gender, age, education, and smoking status ($p>0.05$) between the two groups. Although not significant, apparently male represented the majority of non-obese group while female did in obese group. All participants in both groups had blood glucose level and HbA1c within normal range (Table 1).

Measurement of 8-OHdG in study participants

Table 2 shows that the median (interquartile levels) of 8-OHdG was significantly higher in the obese group compared to controls ($p<0.05$).

Relation between BMI and 8-OHdG in study participants

Spearman correlation analysis shows that BMI and 8-OHdG were significantly and positively correlated ($r= 0.266$, $p=0.045$) (Fig 1).

Discussion

Normal cellular metabolism as well as pathophysiological process could result in production of oxidant species [22]. Oxidative damage

Table 1: Summarizes data for characteristics of the study participants.

Variable	Obese (n=23)	Non-obese (n=36)	p-value
Male, n (%)	9 (39.1)	21 (58.3)	0.150
Age, years	46.8±9.1	42.8±11.6	0.159
Weight, Kg	87.0±14.9	67.2±9.3	0.000*
Height, cm	153.4±20.5	163.8±7.1	0.007*
BMI, kg/m^2	35.7±6.1	25.7±2.4	0.000*
Education, University or high Illiterate	 6 (26.1) 8 (34.8)	 16 (44.4) 10 (27.8)	 0.276
Smoking, n (%)	5 (21.7)	5 (13.9)	0.433
Blood glucose, mmol/l	5.22±0.40	5.74±0.34	0.349
Hb A1c, %	4.7±0.30	5.2±0.24	0.201

Data are represented as mean± SD or number (%)
BMI: Body mass index; HbA1c: glycosylated hemoglobin

Table 2: Comparison of serum 8-OHdG levels between obese and non-obese groups.

	Obese		Non-obese	
	Means ± SD	Median (IQR)	Means ± SD	Median (IQR)
8OHdG (ng/ml)	0.638±0.10*	0.51 (0.32–0.71)**	0.405±0.05	0.325 (0.19–0.49)

Data presented as mean ± SD, median (IQR).
8-OHdG: 8-hydroxy-2`-deoxyguanosine; IQR: interquartile range.
* $p=0.025^{*}$, ** $p= 0.023$

https://doi.org/10.46389/rjd-2020-1053
© 2020 The Authors

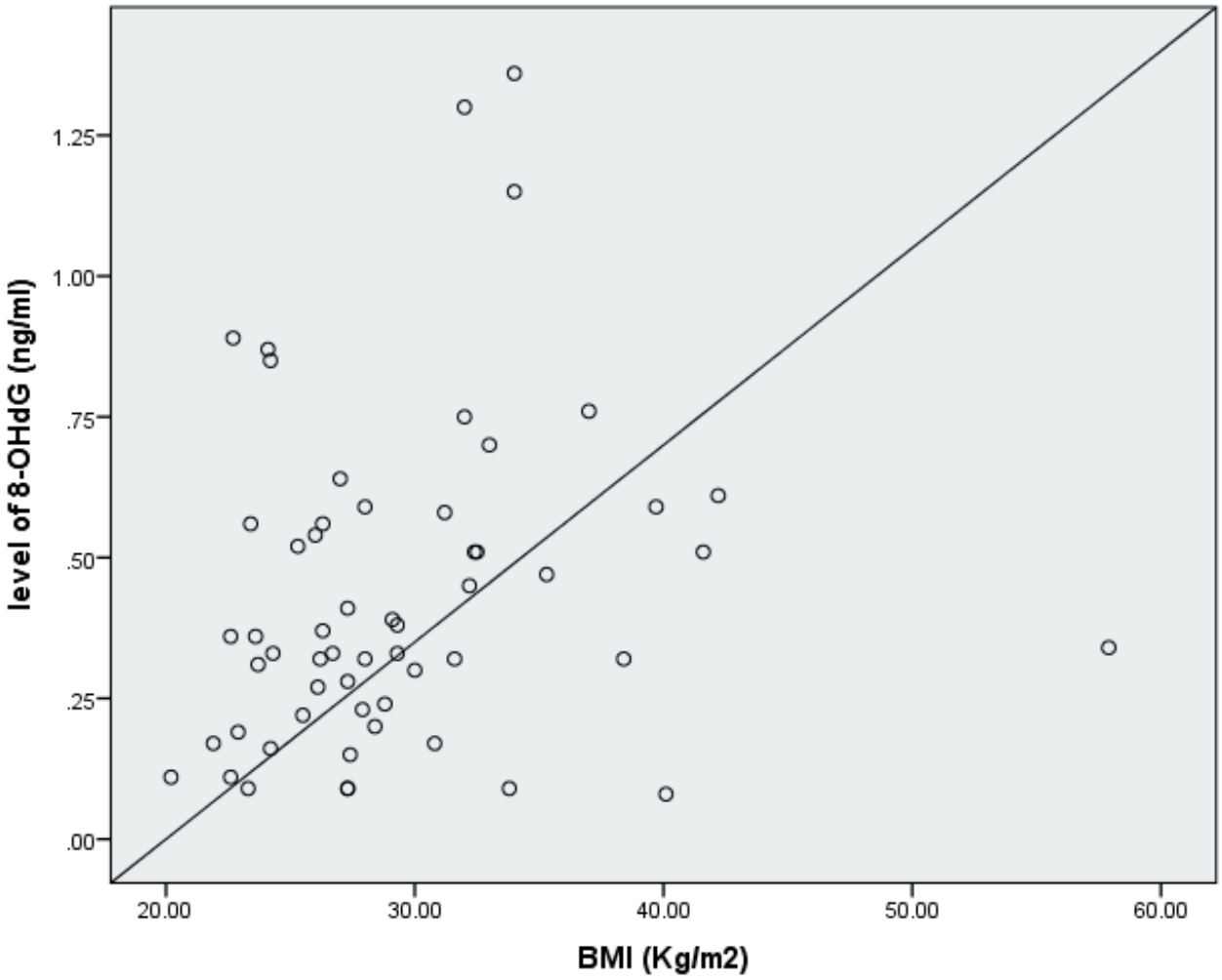


Figure 1: Positive correlation between level of serum 8-OHdG (ng/ml) and BMI (Kg/m^2) in all participants (r = 0.266*, p = 0.045)

to DNA is the most critical modification compared to other cellular biomolecules notably: proteins and lipids [23]. 8-OHdG is a DNA guanine residue modification produced by ROS [24].

We have shown here that the median (interquartile levels) of serum 8-OHdG was significantly higher in the obese subjects in comparison with controls ($p<0.05$). Moreover, data from present study show a statistically significant positive correlation between level of serum 8-OHdG (ng/ml) and BMI (Kg/m^2) in study participants.

Obesity is a multifactorial complex progressive disorder that characterized by excessive fat accumulation as a result of surplus caloric intake or sedentary lifestyle [25]. In obese individuals, the adipose tissue is a source for ROS that released into peripheral blood [26]. The increased release of free fatty acids from over-accumulated fat leads to activation of NADPH oxidase in adipocytes and ultimately in ROS production. NAD phosphate (NADPH) oxidase is an enzyme involved in nutrient-based ROS generation [27]. Direct reaction of ROS with DNA produces a multiplicity of modifications of base and sugar in DNA which if left unrepaired, can lead to diseases [28, 29]. Also, the DNA damage could induce inflammation in visceral adipose tissue which result in development of systemic insulin resistance and lipids dysregulation that accelerate various disease states in individuals with obesity [30]. In support for this approach, Dandona P et al., reported significant reduction in ROS and oxidative stress occurred in nondiabetic obese subjects after short period of dietary restriction and weight loss [31].

In agreement with our results, a study reported a significant increase in serum 8-OHdG in obese subjects (848.5G103 pg/ml;

p<0.001) compared with the lean subjects (196.5G327 pg/ml) [32]. Another study reported that patients with obesity present increased concentrations of oxidative stress by-products such 8-oxo-7,8-dihydro-2'-deoxyguanosine, which is DNA oxidized and highly mutagenic base [33]. In support to these findings, two studies reported high level of mean DNA damage (measured by the comet assay) in obese women compared with non-obese women [34, 35].

Interestingly, Kocael A et al [36] reported significantly (p<0.001) decreased levels of serum 8-OHdG in morbidly obese adults (BMI>40 Kg/m^2) after six months of bariatric surgery which concurrent with efficient weight loss. Authors concluded that the systemic oxidative DNA damage was increased by the morbid obesity in spite that pre- and postoperative serum levels did not correlate with their corresponding weight and BMI. Another study reported increased serum 8-oxo-deoxyguanosine in morbid obese patients when compared to controls and after one year of bariatric surgery [37].

In contrary to our findings, Donmez-Altuntas H et al., [38] reported that plasma concentrations of 8-OHdG in obese subjects were lower compared with normal weight controls (p<0.05). In addition, authors did not observe significant correlation between plasma concentrations of 8-OHdG and BMI (p>0.05). They attributed their findings to efficient repair of the 8-OHdG lesions by base-excision repair pathway. Also, Sakano et al., [39] reported that urinary 8-OHdG was negatively associated with BMI in healthy Japanese subjects.

Conclusions

We have shown here that the serum level of an oxidative DNA damage marker, 8-OHdG, was increased in healthy obese individuals compared to non-obese. There was a significant positive association between DNA damage and BMI. Since weight loss is a manageable and modifiable factor, the study findings suggest that weight loss for obese individuals could reduce DNA damage and oxidative stress which underlie the pathogenesis of obesity-associated metabolic disorders.

Conflict of Interest

The authors declare no conflict of interest.

References

1. Flier JS. Obesity wars: molecular progress confronts an expanding epidemic. *Cell*. 116(2):337–50, 2004.
2. Andreassi MG, Barale R, Iozzo P, Picano E. The association of micronucleus frequency with obesity, diabetes and cardiovascular disease. *Mutagenesis*. 26(1):77–83, 2011. doi: 10.1093/mutage/geq077
3. Ahmed MH, Ali YA, Awadalla H, Elmadhoun WM, Noor SK, Almobarak AO. Prevalence and trends of obesity among adult Sudanese individuals: Population based study. *Diabetes Metab Syndr*. 11(2):S963–S967, 2017. doi: 10.1016/j.dsx.2017.07.023
4. Ogden CL, Carroll MD, Kit BK, Flegal KM. Prevalence of obesity among adults: United States, 2011-2012. *NCHS Data Brief*. 131:1–8, 2013.
5. Lee JY, Lee YR, Kim HR, Myong JP, Kang MY. Trends in Obesity Prevalence by Occupation Based on Korean National Health and Nutrition Examination Survey From 1998 to 2015. *Saf Health Work*. 11(1):97–102, 2020. doi: 10.1016/j.shaw.2019.08.003
6. Flegal KM, Graubard BI, Williamson DF, Gail MH. Excess deaths associated with underweight, overweight, and obesity. JAMA. 293(15):1861–7, 2005.
7. Musaad S, Haynes EN. Biomarkers of obesity and subsequent cardiovascular events. *Epidemiol Rev*. 29:98–114, 2007.
8. Andreassi MG, Barale R, Iozzo P, Picano E. The association of micronucleus frequency with obesity, diabetes and cardiovascular disease. *Mutagenesis*. 26(1):77–83, 2011. doi: 10.1093/mutage/geq077
9. Auten RL, Davis JM. Oxygen toxicity and reactive oxygen species: the devil is in the details. *Pediatr Res*. 66(2):121–7, 2009. doi: 10.1203/PDR.0b013e3181a9eafb
10. De Bont R, van Larebeke N. Endogenous DNA damage in humans: a review of quantitative data. Mutagenesis. 19(3):169–85, 2004.
11. Suzuki K, Ito Y, Ochiai J, Kusuhara Y, Hashimoto S, Tokudome S, Kojima M, Wakai K, Toyoshima H, Tamakoshi K, Watanabe Y, Hayakawa N, Maruta M, Watanabe M, Kato K, Ohta Y, Tamakoshi A. JACC Study Group. Relationship between obesity and serum markers of oxidative stress and inflammation in Japanese. *Asian Pac J Cancer Prev*. 4(3):259–66, 2003.
12. Schulze PC, Lee RT. Oxidative stress and atherosclerosis. *Curr Atheroscler Rep*. 7(3):242–8, 2005.
13. Usman M, Volpi EV. DNA damage in obesity: Initiator, promoter and predictor of cancer. *Mutat Res*. 778:23–37, 2018. doi: 10.1016/j.mrrev.2018.08.002
14. Vincent HK, Taylor AG. Biomarkers and potential mechanisms of obesity-induced oxidant stress in humans. *Int J Obes (Lond)*. 30(3):400–18, 2006.
15. Vincent HK, Innes KE, Vincent KR. Oxidative stress and potential interventions to reduce oxidativestress in overweight and obesity. *Diabetes Obes Metab*. 9(6):813–39. 2007.
16. Makromolekula N. New Markers of Oxidative Damage to Macromolecules. JMB. 27: 1–16, 2008. doi: 10.2478/v10011-007-0049-x

 https://doi.org/10.46389/rjd-2020-1053 © 2020 The Authors

17. Gray K, Bennett M. Role of DNA damage in atherosclerosis–bystander or participant? Biochem Pharmacol. 82(7):693–700, 2011. doi: 10.1016/j.bcp.2011.06.025.
18. Vincent HK, Taylor AG. Biomarkers and potential mechanisms of obesity-induced oxidant stress in humans. *Int J Obes (Lond)*. 30(3):400–18, 2006.
19. Al-Aubaidy HA, Jelinek HF. Oxidative DNA damage and obesity in type 2 diabetes mellitus. *Eur J Endocrinol*. 164(6):899–904, 2011. doi: 10.1530/EJE-11-0053
20. Koide S, Kinoshita Y, Ito N, Kimura J, Yokoyama K, Karube I. Determination of human serum 8-hydroxy-2'-deoxyguanosine (8-OHdG) by HPLC-ECD combined with solid phase extraction (SPE). *J Chromatogr B Analyt Technol Biomed Life Sci*. 878(23):2163–7, 2010. doi: 10.1016/j.jchromb.2010.06.015
21. Obesity: preventing and managing the global epidemic. Report of a WHO consultation. *World Health Organ Tech Rep Ser*. 894:i–xii, 1–253, 2000.
22. Bhattacharyya A, Chattopadhyay R, Mitra S, Crowe SE. Oxidative stress: an essential factor in the pathogenesis of gastrointestinal mucosal diseases. *Physiol Rev*. 94(2):329–54, 2014. doi: 10.1152/physrev.00040.2012
23. Cooke MS, Evans MD, Dove R, Rozalski R, Gackowski D, Siomek A, Lunec J, Olinski R. DNA repair is responsible for the presence of oxidatively damaged DNA lesions in urine. *Mutat Res*. 574(1–2):58–66, 2005.
24. Shin CS, Moon BS, Park KS, Kim SY, Park SJ, Chung MH, Lee HK. Serum 8-hydroxy guanine levels are increased in diabetic patients. *Diabetes Care*. 24(4):733–7, 2001.
25. Nijhawan P, Arora S, Behl T. Intricate role of oxidative stress in the progression of obesity. *Obesity Medicine*. 15: 100125, 2019.
26. Marseglia L, Manti S, D'Angelo G, Nicotera A, Parisi E, Di Rosa G, Gitto E, Arrigo T. Oxidative stress in obesity: a critical component in human diseases. *Int J Mol Sci*. 16(1):378–400, 2014. doi: 10.3390/ijms16010378
27. Matsuda M, Shimomura I. Increased oxidative stress in obesity: implications for metabolic syndrome, diabetes,hypertension, dyslipidemia, atherosclerosis, and cancer. *Obes Res Clin Pract*. 7(5):e330–41, 2013.
28. Andreassi MG. DNA damage, vascular senescence and atherosclerosis. *J Mol Med (Berl)*. 86(9):1033–43, 2008. doi: 10.1007/s00109-008-0358-7
29. Noren Hooten N, Ejiogu N, Zonderman AB, Evans MK. Association of oxidative DNA damage and C-reactive protein in women at risk for cardiovasculardisease. *Arter ioscler Thromb Vasc Biol*. 32(11):2776–84, 2012. doi: 10.1161/ATVBAHA.112.300276
30. Shimizu I, Yoshida Y, Suda M, Minamino T. DNA damage response and metabolic disease. *Cell Metab*. 20(6):967–77, 2014. doi: 10.1016/j.cmet.2014.10.008
31. Dandona P, Mohanty P, Ghanim H, Aljada A, Browne R, Hamouda W, Prabhala A, Afzal A, Garg R. The suppressive effect of dietary restriction and weight loss in the obese on the generation of reactive oxygen species by leukocytes, lipid peroxidation, and protein carbonylation. *J Clin Endocrinol Metab*. 86(1):355–62, 2001.
32. Al-Aubaidy HA, Jelinek HF. Oxidative DNA damage and obesity in type 2 diabetes mellitus. *Eur J Endocrinol*. 164(6):899–904, 2011. doi: 10.1530/EJE-11-0053
33. Cerda C, Sanchez C, Climent B, Vazquez, A, Iradi A, El Amrani F; Bediaga A, Saez GT. Oxidative stress and DNA damage in obesity-related tumorigenesis. *Adv. Exp. Med. Biol*. 824: 5–17, 2014.
34. Zaki M, Basha W, El-Bassyouni HT, El-Toukhy S, Hussein T. Evaluation of DNA damage profile in obese women and its association to risk of metabolic syndrome, polycystic ovary syndrome and recurrent preeclampsia. *Genes Dis*. 5(4):367–373, 2018. doi: 10.1016/j.gendis.2018.03.001. eCollection 2018 Dec
35. Włodarczyk M, Jabłonowska-Lietz B, Olejarz W, Nowicka G. Anthropometric and Dietary Factors as Predictors of DNA Damage in Obese Women. *Nutrients*. 10(5). pii: E578, 2018. doi: 10.3390/nu10050578
36. Kocael A, Erman H, Zengin K, Kocael PC, Korkmaz GG, Gelisgen R, Taskin M, Ersan Y, Uzun H. The effects on oxidative DNA damage of laparoscopic gastric band applications in morbidly obese patients. *Can J Surg*. 57(3):183–7, 2014.
37. Monzo-Beltran L, Vazquez-Tarragón A, Cerdà C, Garcia-Perez P, Iradi A, Sánchez C, Climent B, et al. One-year follow up of clinical, metabolic and oxidative stress profile of morbid obese patients after laparoscopic sleeve gastrectomy. 8-oxo-dG as a clinical marker. *Redox Biol*. 12:389–402, 2017. doi: 10.1016/j.redox.2017.02.003
38. Donmez-Altuntas H, Sahin F, Bayram F, Bitgen N, Mert M, Guclu K, Hamurcu Z, Arıbas S, Gundogan K, Diri H. Evaluation of chromosomal damage, cytostasis, cytotoxicity, oxidative DNA damage and their association with body-mass index in obese subjects. *Mutat Res Genet Toxicol Environ Mutagen*. 771:30–6, 2014. doi: 10.1016/j.mrgentox.2014.06.006. Epub 2014 Jun 28.
39. Sakano N, Takahashi N, Wang DH, Sauriasari R, Takemoto K, Kanbara S, Sato Y, Takigawa T, Takaki J, Ogino K. Plasma 3-nitrotyrosine, urinary 8-isoprostane and 8-OHdG among healthy Japanese people. *Free Radic Res*. 43(2):183–92, 2009. doi: 10.1080/10715760802663124

© 2020 The Authors

https://doi.org/10.46389/rjd-2020-1054
www.rjdnmd.org

Rom J Diabetes Nutr Metab Dis
2020; volume 27, issue 4, pages 372-380

Original Research

Relationship of angiotensin converting enzyme (I/D) polymorphism (rs4646994) and ischemic heart disease in Iraqi patients with type 2 diabetes mellitus

Raghda N. Hemeed[1], Fadhil J. Al-Tu'ma[2], Dhafer A. F. Al-Koofee[3*], and Ahmed H. Al-Mayali[4], Abdolmajid Ghasemian[4]

[1, 2] Department of Biochemistry, College of Medicine, University of Kerbala, Iraq
[3] Department of Clinical Laboratory Science, Faculty of Pharmacy, University of Kufa, Najaf, Iraq
[4] Department of Internal Medicine, College of Medicine, University of Kerbala, Iraq

*Correspondence to: Dhafer A. F. Al-Koofee, Department of Clinical Laboratory Science, Faculty of Pharmacy, University of Kufa, Najaf, Iraq, E-mail: dhafera.faisal@uokufa.edu.iq, Phone +9647805448773

Received: 3 June 2020 / Accepted: 5 September 2020

Abstract

Insertion deletion (I/D) polymorphism (rs4646994) in the angiotensin-converting enzyme (ACE) has a substantial effect on Coronary Heart Disease (CHD). The objective of this study was to investigate the association between the *ACE* gene polymorphism and CHD in Iraqi patients with and without Type-2 Diabetes Mellitus (T2DM). The amplification of an Alu repetitive element in an intron of the *ACE* has shown three potential genotypes of I/I and D/D as homozygous, and I/D as heterozygous. A total of 217 individuals participated in this study, and they were divided into three groups; Group 1 included 86 patients who had CHD with T2DM, Group 2 included 78 patients who had CHD without T2DM, and Group 3 included 53 age and sex-matched healthy individuals (as a control group). Genotyping of *ACE* (I/D) gene was performed using polymerase chain reaction (PCR) technique. Our data showed a significantly high D/D genotype frequency in the *ACE* in CHD patients compared to the healthy individuals (OR=4.39, CI 1.57223–12.2749, P<0.0048), whereas I/D genotype was not affected (OR=1.1009, CI 0.5540–2.1878). This suggested that the D/D genotype is an independent risk factor for CHD in Iraqi patients with and without T2DM. We concluded that the D/D genotype is implicated as a risk factor for CHD patients, in the Iraqi population. However, a larger sample size is needed to monitor the CHD patients and validate this study.

Keywords: Angiotensin converting enzyme, Coronary heart disease, Ischemic heart disease 2 Diabetes Mellitus.

Introduction

In coronary heart disease (CHD), fat accumulates at the end of the atrium due to an unusual metabolism of this substance. These precipitated fats cause reduction of the arteries' cavity, retarded blood flow, and consequently leads to ischemic heart failure along with clinical symptoms such as angina. Because of the high morbidity and death rate, CHD has become one of the most dangerous cardiovascular diseases threatening people's lives [1, 2]. Approximately, one third or more of all deaths in people of 35 years old and older are due to this disease [3]. The most common cause of ischemia is atherosclerosis, which is the consequence of dyslipidemia, causing a local decrease in blood-flow through the heart muscle, as well as insufficient heart muscle perfusion provided by the coronary artery [4]. It occurs to individuals of all ages, but is more common in the elderly, with males being more susceptible than females. However, other common risk factors are smoking, family history, high blood pressure, excessive weight gain, diabetes, high alcohol consumption, little or no exercise, stress, and high blood fat [5]. Symptom

© 2020 The Authors. Romanian Journal of Diabetes, Nutrition and Metabolic Diseases published by Sanatatea Press Group on behalf of the Romanian Society of Diabetes Nutrition and Metabolic Diseases. This is an open access article under the terms of the Creative Commons Attribution License (CC-BY-NC-ND 3.0).

of Ischemic Heart Disease (IHD) includes angina (severe chest pain during exertion) and heart palpitation due to decrease or lack of exercise. Diagnosis of the IHD is done through an electrocardiogram, cardiac stress test, blood tests, or coronary angiography. For symptomatic patients, a stress echocardiogram is used to diagnose obstructive coronary artery disease [6]. The patient's age, gender, coronary risk factors, and the nature of chest pain are also important factors that determine the risk of CHD and facilitate the diagnosis. Hence, the ECG exercise test is a diagnostic test that needs to be performed accurately [7]. Partial or complete blocked arteries cause angina or heart attack, respectively, due to the gradual death of heart cells [8]. The actual numbers of these anomalies vary from one country to other [9]. Each year, approximately 790,000 adults suffer from myocardial infarction (MI), and 210,000 of them suffer from frequent heart attacks [10]. Somatic angiotensin-I converting enzyme (sACE) plays an essential role in regulating the blood pressure and electrolyte fluid balance. It is a zinc protease that cleaves angiotensin-I (AngI), bradykinin, and a large group of other signal peptides [11]. The ACE is expressed in cells in the bone marrow and encoded angiotensin-converting enzyme (ACE). It converts angiotensin I to angiotensin II active peptide, leading to increased hematopoietic stem cells [12]. Single nucleotide polymorphisms (SNPs) of renin-angiotensin system (RAS) genes such as ACE, has been shown to be linked strongly to cardiovascular disease (CVD) [13]. In CVD, one of the most extensively studied genes was the ACE and I/D polymorphism of ACE gene that strongly associated with the ACE activity [14].

Materials & Methods

Subjects

This study was designed as a case-control survey comprising of 217 male subjects classified into three groups. The group I had 86 patients who had IHD with T2DM, the group II had 78 patients having IHD without T2DM, and the group III had 53 apparently-healthy individuals without any diseases, which were used as a control group. Samples were collected from Al-Hussein Medical City, Kerbala from December 1/2018 to July 31/2019. The biochemical parameter determinations and genetic analysis were performed in the Department of Biochemistry, College of Medicine, University of Kerbala and Laboratory of Al-Hussein Medical City. The exclusion criteria were: (1) Female; (2) Type 1 diabetes mellitus; (3) Patients treated with catheterization; (4) Children. The inclusion criteria were as follows: (1) The selected patients diagnosed with one of the following diseases: myocardial infarction (MI) with T2DM, MI without T2DM, unstable angina with T2DM, unstable angina without T2DM; (2) Adult male patients; (3) Before performing catheterization; (4) With or without risk factor: smoking, family history, hypertension and T2DM. Informed consent was taken from all subjects according to the Ethical Committee of the Kerbala Medical College, who approved the study. Blood was drawn (5 ml) through vein puncture from all participants. The collected blood was divided into three parts: (1) One milliliter of blood used for molecular analysis (DNA extraction), was collected in the EDTA containing tube, (2) One milliliter used for HbA1c analysis in the EDTA containing tube, (3) Three milliliters of blood collected in a gel tube for biochemical analysis.

The *ACE* (I/D) polymorphism (rs4646994) detection by polymerase chain reaction

DNA was extracted from the blood samples by the genomic DNA extraction Kit (Geneaid Biotech Ltd., UK), according to the manufacturers' protocol. Concentration and purity of isolated DNA was measured by a BioDrop (UK). The existence of the I and D alleles of the ACE was detected by PCR, according to the essay described by Zmorzynski *et al.* [12)] The sequence of forward primer was 5'-CTG GAG ACC ACT CCC ATC CTT TCT-3', and the reverse primer was 5'-GAT GTG GCC ATC ACA TTC GTC AGAT-3'. PCR reaction was performed with 100 ng DNA in a final volume of 25 µl, containing 12.5 µl mastermix (Promega, Madison, WI, USA), 1 µl of each primer

© 2020 The Authors

(10 pmol). The PCR program for determining the genotype of the ACE (I/D) (rs4646994) was done in a thermal cycler (Biometra, Germany), with 95˚C initial denaturation for 5 minutes, and 35 cycles of denaturation at 95˚C for 30 seconds, annealing at 60˚C for 30 seconds, and extension at 72˚C for 40 seconds, and finally, one cycle at 72˚C for 5 minutes. Lastly, 7 µl of amplicon was separated by electrophoresis on a 2% agarose gel, containing safe stain and finally visualized by a transilluminator. The I allele manifested as a 490 bp band, while the D allele was seen as a 190 bp band of DNA, but I/D genotype, illustrated as two bands, appeared at 490 bp and 190 bp as demonstrated in Figure 1.

D/D genotype showed a single band with 190 bp, lane 2,4,7 homozygous; I/I genotype had a single band with 490 bp, lane 1,3,5,9 homozygous; and I/D genotype demonstrated two bands 190 and 490 bp, lane 6, 8 heterozygous; L is the indicated ladder.

Statistical analysis

A student t-test was applied to biochemical variables, in comparison with IHD patients with polymorphism, and Chi-square test for categorical variables, using SPSS v.25. Fischer's exact test was used to assess the relationship of the ACE I/D polymorphism with the risk factor. Also, the quantitative information was shown as the frequency or percentage values. Online website (https://wpcalc.com/en/equilibrium-hardy-weinberg/) was used to evaluate the Hardy-Weinberg equilibrium (HWE) and the Chi square test was performed for determining the genotype frequencies in all groups. The statistical significance was considered at $p<0.05$. The analysis of variance (ANOVA) was performed to analyze the lipid profile parameters.

Results

164 males with CHD, with and without T2DM, participated in the study, with a mean age of 55.5 years, and 53 healthy individuals with a mean age 46.5 years were considered as a control group. Clinical characteristics at the time of diagnosis are listed in Table 1. All 217 individuals were examined successfully for genotype analysis in this work. The genotype frequencies of all groups were confirmed by HWE test, as I/I, I/D and D/D polymorphism in controls were 54.7%, 36% and 9.5% respectively and along with CHD, in T2DM patients, were 52%, 16.3% and 31.4% respectively, while CHD without T2DM were 84.6, 2.56, and 12.8, respectively, as shown in Table 2. No statistical significance was observed in allele frequency between control and CHD patients' groups. The allele frequencies for both groups are listed in Table 3. The frequency of I allele appeared higher than D allele in both groups. In addition, an association between CHD with T2DM and D/D genotype was observed; OR =4.3932; $p<0.0048$ as shown in Table 4. Furthermore, an association between CHD without T2DM and I/D genotype was shown; OR= 0.046; $p<0.0001$ as presented in Table 5.

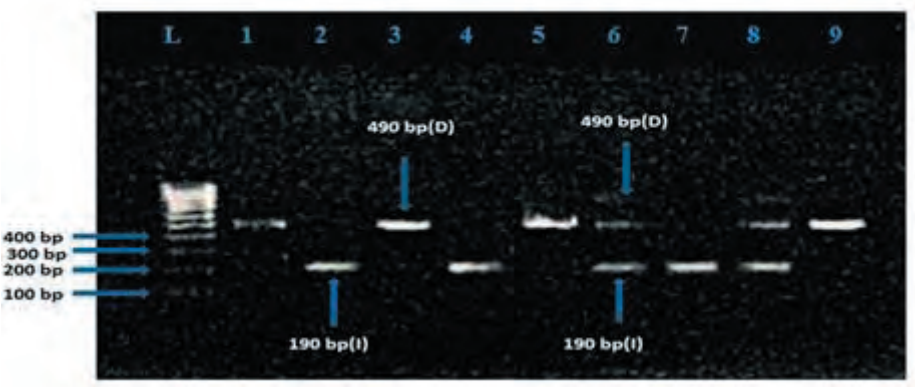


Figure 1: Genotypes of the ACE polymorphism (I/I, I/D and D/D)(rs4646994).

https://doi.org/10.46389/rjd-2020-1054
© 2020 The Authors

Table 1: The characteristics of CHD patients and control group participated in the study.

		I/I homozygous	I/D heterozygous	D/D homozygous
		Control		
Male (N)	53	29	19	5
Age (year)	47	43	49	59
Cholesterol mg/dL	143±30.5	134.99±33.7	154.1±22.8	149.37±25.67
TG mg/dL	77.6±16.9	78.15±17	77.889±16.75	73.3±220.3
HDL mg/dL	38±3	38.4±12.3	37.8±10.1	39.7±16.65
LDL mg/dL	92±3174	9.41±34.11	88.89±31.2	101.5±20.5
VLDL mg/dL	15.52±3.4	15.63±33.4	15.6±3.35	14.67±4.1
		CHD with T2DM		
Male (N)	86	45	14	27
Age (year)	57	54	69	66
Cholesterol (mg/dL)	176.6±44.3	188.2±45.9	148.7±33.6	171.8±40.1
TG (mg/dL)	187.26±12	201.5±91.8	125.4±19.4	195.6±83.6
HDL (mg/dL)	38.4±12.2	37.47±12.2	46.6±9.7	35.9±12.1
LDL (mg/dL)	135.2±33.84	137.88±33	141±37.6	127.6±33.16
VLDL (mg/dL)	31.4±11	34±13	25.47±5.7	30.2±7.8
		CHD without T2DM		
Male (N)	78	66	2	10
Age (year)	54	55	63	47
Cholesterol (mg/dL)	162.4±24.5	165.45±24.2	144.5±12.1	145.9±21.1
TG (mg/dL)	162.4±72.4	174±72.7	99.5±1.4	98.8±17.2
HDL (mg/dL)	34.7±6.2	34.9±6.22	31.8±4.3	34.2±6.5
LDL (mg/dL)	141.6±49.2	148±48.7	93±29.2	109.8±39.4
VLDL (mg/dL)	34.3±14.5	36.8±14.31	21.4±2	20.5±3.7

Furthermore, the biochemical parameters were assessed in the control group, in addition to both groups of CHD patients, with and without T2DM, as demonstrated in table 6.

Discussion

Coronary heart disease is the most common polygenic disease that includes complex interactions between genes and several biochemical risk factors, making it the leading cause of death in many countries [15]. RAS has been recognized as a significant pathway for regulation of blood pressure, in addition to kidney function [16]. Therefore, the main aim of this study was to investigate the association between the ACE gene polymorphism and CHD in Iraqi patients, with and without T2DM. According to the World Health Organization (WHO), CHD represents the first cause of death in Iraq with 18.5% of total deaths. An extensive study suggested that polymorphisms in the constituents of RAS are significant in the development and consequences of CHD, within T2DM populations [17, 18], in addition to their role in atherosclerotic disease and related vascular complications [19]. The variation of ACE I/D in the intron 16 was shown to be implicated in the risk of CHD, and the frequency of allele deletion (DD) has been found to be higher in CHD with T2DM patients, relative to those carry I/I or I/D alleles (OR=3.48, p<0.02;

© 2020 The Authors

Table 2: The Hardy-Weinberg equilibrium for the ACE (I/D) polymorphism (rs4646994) in CHD patients and control groups, according to the expected and observed values

rs4646994	I/I	I/D	D/D	Total (N)	HWE p-Value & X^2
Control					
Observed	29 (54.7)	19 (36)	5 (9.5)	53	0.47
Expected	28	21	4		0.51
MAF	0.27				
CHD with T2DM					
Observed	45 (52)	14 (16.3)	27 (31.4)	86	0.0001
Expected	31.4	41.1	13.4		37.41
MAF	0.4				
CHD without T2DM					
Observed (%)	66 (84.6)	2 (2.56)	10 (12.8)	78	0.0001
Expected	57.6	18.9	1.6		62.364
MAF	0.14				
	SNP exact test for Hardy-Weinberg equilibrium (n=217)				
	N I/I	**N I/D**	**N D/D**	**N I**	**N D**	**p-Value**
All Subjects	140	35	42	315	119	<0.0001

MAF: Minor allele frequency

Table 3: The comparison of the ACE I/D allele frequencies among CHD patients and control groups

Allele	Control N (%) Total N=53	CHD Patients N (%) Total N=164	p-value
I	**77 (72.6)**	**238 (72.5)**	**0.9871**
D	**29 (27.4)**	**90 (27.4)**	
Total	**106 (100)**	**328 (100)**	

OR=4.39, p<0.0048, respectively). No statistical significance was observed for those who have CHD without T2DM (OR=0.878, p<0.8; OR=1.4, p value<0.55, respectively). The ACE allele frequencies in the healthy subjects were 72.6% for the I allele and 27.4% for the D allele, while the frequencies in the CHD group were 72.5% and 27.4% for the I and D alleles, respectively. Our results are consistent with other studies related to polymorphism at the same position [20, 21], whereas, Turkish and Gaza studies are not in line with our results [18, 22]. The main reasons are differences in both cases and healthy groups' selection criteria, such as age, BMI, hypertension and others, in addition to the risk of heritable factors in the investigated samples, bewildered by the little information of parameters used. The usual factors like diabetes, obesity, and dyslipidemia have been reported in relation to the ACE gene in many issues [23]. Both, metabolic process and genetic factors, are appearing to be linked to severity, and susceptibility to, CHD [17]. In low- and middle-income countries, CHD deaths accounted for 80% of all deaths [24].

In general, the increase in age is associated with abnormalities in many arteries as there are a number of age-dependent changes in the structural components of the artery, which will lead to an increase in the number of elderly patients with heart disease [25]. Our results are in alignment with previous studies that reported on lipid metabolism disorders, suggesting an

https://doi.org/10.46389/rjd-2020-1054
© 2020 The Authors

Table 4: The association between the ACE (I/D) genotypes and the risk of CHD with T2DM

Genotype	Control N= 53	CHD with T2DM N= 86	OR (95% CI)	p value
Co-dominant				
II	29	45	1.00	
ID	19	14	0.4749 0.2064 to 1.0926	0.0798
DD	5	27	3.4800 1.2029–0.0675	0.0214
Dominant				
ID+DD	24	41	1.1009 0.5540–2.1878	0.7838
Recessive				
II+ID	48	59	1.00	
DD	5	27	4.3932 1.57231–2.2749	0.0048
Over dominant				
II+DD	34	72	1.00	
ID	19	14	0.3480 0.1561–0.7758	0.0099

Table 5: The association between the ACE I/D genotypes and risk of CHD without T2DM

Genotype	Control N=53	CHD without T2DM N= 78	OR (95% CI)	P value
Co-dominant				
II	29	66	1.00	
ID	19	2	0.046 0.0101–0.2117	0.0001
DD	5	10	0.8788 0.2758–2.8003	0.8270
Dominant				
ID+DD	24	12	0.2197 0.0969–0.4984	0.0003
Recessive				
II+ID	48	68	1.00	
DD	5	10	1.4118 0.4536–4.3938	0.5516
Over dominant				
II+DD	34	76	1.00	
ID	19	2	0.0471 0.0104–0.2136	0.0001

© 2020 The Authors

Table 6: Biochemical characteristics of control and CHD with and without T2DM

Parameters	Mean±SE	Group Comparison	P-Value
HDL (mg/dL)	Control	CHD with T2DM	0.928
	38.3±1.62	CHD without T2DM	.055
	CHD with T2DM		
	38.5±1.3	CHD without T2DM	.023
	CHD without T2DM		
	34.75±0.7		
LDL (mg/dL)	Control	CHD with T2DM	.000
	92±4.36	CHD without T2DM	.000
	CHD with T2DM		
	135±3.65	CHD without T2DM	.300
	CHD without T2DM		
	141.6±5.57		
VLDL (mg/dL)	Control	CHD with T2DM	.000
	15.52±0.46	CHD without T2DM	.000
	CHD with T2DM		
	31.4±1.2	CHD without T2DM	.090
	CHD without T2DM		
	34.38±1.64		
TG (mg/dL)	Control	CHD with T2DM	.000
	77.6±2.32	CHD without T2DM	.000
	CHD with T2DM		
	187.25±9.2	CHD without T2DM	.023
	CHD without T2DM		
	162.4±8.2		
CH (mg/dL)	Control	CHD with T2DM	0.000
	143.2±4.2	CHD without T2DM	0.002
	CHD with T2DM		
	176.6±4.7	CHD without T2DM	0.01
	CHD without T2DM 162.4±2.8	CHD without T2DM	

Bold values are statistically significant.

essential role in atherosclerosis progress in CHD patients. They appeared elevated in all lipid constituents except HDL that came with low levels. In most countries, dyslipidemia is considered important as a dynamic risk factor for atherosclerosis, and confirmed for CHD [26] where lipid profiles' disturbances in plasma lead to an increase susceptibility, causing CHD [27]. A number of mechanisms are suggested that clarify the strong consequences of lipid profile on the CHD, depending on the physiological role of lipids [28]. HDL carries about 20% of the total plasma cholesterol, transporting excess cholesterol from the arterial wall's foam macrophages to

https://doi.org/10.46389/rjd-2020-1054
© 2020 The Authors

the liver [29], causing the outflow of cholesterol from peripheral cells. A potent association exists between low levels of HDL and the risk for atherosclerosis. On the other hand, LDL, as a major atherogenic lipoprotein, supports cholesterol accumulation in the vessel wall, causing a hindrance of blood flow.

Our work is relatively limited due to the small sample size. We strongly suggest further investigations on a larger number of cases that may validate the significance of ACE I/D polymorphism in the pathobiology of CHD.

Conclusions

So, we have concluded that D/D genotype of the ACE polymorphism (rs4646994) is associated with more than 4-fold higher predisposition to CHD. However, the dyslipidemia was also implicated in the severity of CHD.

Acknowledgement

We would like to thank all the staff of laboratory of Al-Hussein Medical City and the participants of both healthy and CHD groups for their cooperation.

Conflicts of interest

The authors declare no conflict of interest.

References

1. Lala A, Desai AS. The role of coronary artery disease in heart failure. Heart failure clinics.10(2):353–65, 2014.
2. Piccolo R, Giustino G, Mehran R, Windecker S. Stable coronary artery disease: revascularisation and invasive strategies. *The Lancet*. 386(9994):702–13, 2015.
3. Wu C, Woo JG, Zhang N. Association between urinary manganese and blood pressure: Results from National Health and Nutrition Examination Survey (NHANES), 2011–2014. *PloS one*.12(11):e0188145, 2017.
4. Hamirani YS, Nasir K, Blumenthal RS, Takasu J, Shavelle D, Kronmal R, et al. Relation of mitral annular calcium and coronary calcium (from the Multi-Ethnic Study of Atherosclerosis [MESA]). *The American journal of cardiology*. 107(9):1291–4, 2011.
5. Finegold JA, Asaria P, Francis DP. Mortality from ischaemic heart disease by country, region, and age: statistics from World Health Organisation and United Nations. *International journal of cardiology*. 168(2):934–45, 2013.
6. Al-Jubawii AA, Al-Humairi AK, Ali MH. Incidence of Ischemic Heart Disease among Patients with Mitral Annulus Calcification in Babylon Province. *Journal of University of Babylon*. 24(5):1421–32, 2016.
7. Kivimäki M, Nyberg ST, Batty GD, Fransson EI, Heikkilä K, Alfredsson L, et al. Job strain as a risk factor for coronary heart disease: a collaborative meta-analysis of individual participant data. *The Lancet*. 380(9852):1491–7, 2012.
8. Ismael MK, Al-Haleem MR, Salman RS. Evaluation of Anti-Helicobacter pylori Antibodies in A group of Iraqi Patients with Atherosclerosis and Coronary Artery Disease. Iraqi Journal of Science. 2015;56(1A):81–8, 2015.
9. Eman Ghanim Sheet A-H. Prevalence and Risk Factors for Congenital Heart Anomalies Among Hospital Attendees in Mosul City. *Iraqi Academic Scientific Journal* المجلة العراقية للدراسات الطبية. 14(2):229–36, 2015.
10. Fang J, Ayala C, Luncheon C, Ritchey M, Loustalot F. Use of Outpatient Cardiac Rehabilitation Among Heart Attack Survivors - 20 States and the District of Columbia, 2013 and Four States, 2015. MMWR Morbidity and mortality weekly report. 66(33):869–73, 2017.
11. Masuyer G, Yates CJ, Sturrock ED, Acharya KR. Angiotensin-I converting enzyme (ACE): structure, biological roles, and molecular basis for chloride ion dependence. Biological chemistry. 395(10):1135–49, 2014.
12. Zmorzynski S, Szudy-Szczyrek A, Popek-Marciniec S, Korszen-Pilecka I, Wojcierowska-Litwin M, Luterek M, et al. ACE Insertion/Deletion Polymorphism (rs4646994) Is Associated With the Increased Risk of Multiple Myeloma. *Frontiers in Oncology*. 9(44), 2019.
13. Yang C-H, Lin Y-D, Wu S-J, Chuang L-Y, Chang H-W. High order gene-gene interactions in eight single nucleotide polymorphisms of renin-angiotensin system genes for hypertension association study. *BioMed research international*. 454091, 2015.
14. Madhu S. Normoalbuminuric diabetic kidney disease: a distinct entity? : Springer; 2019.
15. Amara A, Mrad M, Sayeh A, Lahideb D, Layouni S, Haggui A, et al. The effect of ACE I/D polymorphisms alone and with concomitant risk factors on coronary artery disease. Clinical and Applied Thrombosis/Hemostasis. 2018;24(1):157–63.
16. Chen Y-H, Liu J-M, Hsu R-J, Hu S-C, Harn H-J, Chen S-P, et al. Angiotensin converting enzyme DD genotype is associated with acute coronary syndrome severity and sudden cardiac death in Taiwan: a case-control emergency room study. *BMC cardiovascular disorders*. 12(1):6, 2012.
17. Al-Jafari AA, Daoud MS, Ataya FS. Renin-angiotensin system gene polymorphisms and coronary artery disease in Saudi patients with diabetes mellitus. *Int J Clin Exp Pathol*. 10(10):10505–14, 2017.
18. El-Malakh RA. Association between angiotensin converting enzyme gene insertion\deletion polymorphism and coronary heart disease in Gaza Strip. Journal of Biomedical Materials Research. 4(3):18–26, 2016.
19. Temel SG, Ergoren MC, Yilmaz I, Oral HB. The use of ACE INDEL polymorphism as a biomarker of coronary artery disease

(CAD) in humans with Mediterranean-style diet. International journal of biological macromolecules. 123:576–80, 2019.
20. Agachan B, Isbir T, Yilmaz H, Akoglu E. Angiotensin converting enzyme I/D, angiotensinogen T174M-M235T and angiotensin II type 1 receptor A1166C gene polymorphisms in Turkish hypertensive patients. *Experimental & molecular medicine*. 35(6):545, 2003.
21. Al-Jebouri MM, Al-Alwani HR. Angiotensin I-Converting Enzyme Gene Polymorphism In Patients With Chronic Renal Failure. 2014.
22. Bayramoglu A, Kurt H, Gunes HV, Ata N, Birdane A, Dikmen M, et al. Angiotensin II type 1 receptor (AT1) gene A1166C is associated with the risk of hypertension. *Genetic testing and molecular biomarkers*. 19(1):14–7, 2015.
23. Volgman AS, Palaniappan LS, Aggarwal NT, Gupta M, Khandelwal A, Krishnan AV, et al. Atherosclerotic cardiovascular disease in South Asians in the United States: epidemiology, risk factors, and treatments: a scientific statement from the American Heart Association. *Circulation*. 138(1):e1–e34, 2018.
24. Gersh BJ, Sliwa K, Mayosi BM, Yusuf S. Novel therapeutic concepts the epidemic of cardiovascular disease in the developing world: global implications. *European heart journal*. 31(6):642–8, 2010.
25. Haller PM, Jager B, Farhan S, Christ G, Schreiber W, Weidinger F, et al. Impact of age on short- and long-term mortality of patients with ST-elevation myocardial infarction in the VIENNA STEMI network. *Wiener klinische Wochenschrift*. 130(5–6):172–81, 2018.
26. Narne P, Ponnaluri KC, Singh S, Siraj M, Ishaq M. Relationship between angiotensin-converting enzyme gene insertion/deletion polymorphism, angiographically defined coronary artery disease and myocardial infarction in patients with type 2 diabetes mellitus. *Journal of the Renin-Angiotensin-Aldosterone System*. 13(4):478–86, 2012.
27. Abess M, Hussain M, Jeddoa Z. *Lipid Profile and Coronary Heart Disease*. 11:188–94, 2017.
28. Ferrari R, Aguiar C, Alegria E, Bonadonna RC, Cosentino F, Elisaf M, et al. Current practice in identifying and treating cardiovascular risk, with a focus on residual risk associated with atherogenic dyslipidaemia. *European Heart Journal Supplements*. 18(suppl C):C2–C12, 2016.
29. Lewis GF, Rader DJ. New insights into the regulation of HDL metabolism and reverse cholesterol transport. *Circulation research*. 96(12):1221–32, 2005.

 https://doi.org/10.46389/rjd-2020-1054 © 2020 The Authors

https://doi.org/10.46389/rjd-2020-1055
www.rjdnmd.org

Rom J Diabetes Nutr Metab Dis
2020; volume 27, issue 4, pages 381-385

Original Research

Successful dietary intervention plan for Hashimoto's thyroiditis: A case study

Nahla Subhi Al-Bayyari

Department of Nutrition and Food Technology, Faculty of Al-Huson University College, Al-Balqa Applied University, Al-Salt, Jordan

*Correspondence to: Nahla Subhi Al-Bayyari, Department of Nutrition and Food Technology, Faculty of Al-Huson University College, Al-Balqa Applied University, Al-Salt 19117, Jordan. E-mail: n.bayyari@bau.edu.jo, Phone: + 00962795767524

Received: 11 July 2020 / Accepted: 4 October 2020

Abstract

Introduction: A 49-year-old obese woman, medically free from any of chronic diseases, was newly diagnosed with Hashimoto's thyroiditis (HT) of unknown cause by the specialized internist endocrinologist. **Methods:** To manage the case, a modified autoimmune Paleo low-calorie diet (1200 kcal) was recommended for 6 months. The anthropometric measurements, body composition, fasting blood glucose, fasting insulin, non-HDL cholesterol, HDL, triglycerides (TG), thyroxine (T4), triiodothyronine (T3), thyroid stimulating hormone (TSH), and thyroid peroxidase (TPO) were measured on basal level and every 30 days until day 180. **Results:** Showed a significant ($p < 0.05$) reduction in body weight, body mass index, waist and hip circumference, waist to hip ratio, fat mass, TG, non-HDL cholesterol, TSH, and TPO, while T3 and T4 remained within normal reference range. Also, there was a significant elevation in the HDL cholesterol level with statistically non-significant ($p > 0.05$) decrease in fasting insulin. **Conclusion:** The diet improves the TSH, TPO, anthropometric, body composition, HDL and non-HDL cholesterol levels. These improvements will help the HT patients to improve their health and quality of life, as well as reduce inflammation, thyroxine treatment dose, and risk for chronic diseases associated with future hypothyroidism.

Keywords: Autoimmune diet, Case report, Hashimoto thyroiditis.

Introduction

Goitrous autoimmune thyroiditis (AITD), or Hashimoto's thyroiditis (HT) was considered as one of the most common chronic human autoimmune diseases responsible for considerable morbidity in women [1] especially if there is genetic susceptibility together with environmental factors [2]. The presence of thyroid autoantibodies (TAbs) against two major thyroid antigens, thyroid peroxidase (TPO) and thyroglobulin (Tg), in the patients' sera is the principal biochemical characteristic of the disease [3]. The disease results in hypothyroidism due to the gradual atrophy of thyroid tissue followed by the invasion of the gland with lymphocytic cells, follicular atrophy, and hyperemia accompanied by oncocytic metaplasia of follicular cells [4]. According to previous studies, antithyroid antibodies were found to be high in patients with undiagnosed celiac disease. However, committing to a six-month gluten free diet reduced antibody titers and showed lower autoimmune response [5]. Furthermore, when patients with celiac disease and hypothyroidism follow a gluten-free diet, they show improvements in food and medications absorption as a result of intestinal healing, thereby needing lower doses of thyroid medication [6].

The effects of a gluten-free diet on patients who have autoimmune disease (ATD) but not celiac disease have not been evaluated yet. In spite that some researchers report observing benefits in patients, this remains controversial.

© 2020 The Authors. Romanian Journal of Diabetes, Nutrition and Metabolic Diseases published by Sanatatea Press Group on behalf of the Romanian Society of Diabetes Nutrition and Metabolic Diseases. This is an open access article under the terms of the Creative Commons Attribution License (CC-BY-NC-ND 3.0).

Also, the new dietary protocol called the autoimmune protocol (AIP) [7] which was used to help people with autoimmune diseases, such as the inflammatory bowel disease is hard to follow for many patients as well as it may not be helpful for all cases of HT. Also, previous studies did not study the effect of AIP diet on HT patient anthropometric and body composition. Therefore, I planned a special diet which is a mixture of Paleo, gluten-free, and vegan diet and examined the effect of this diet on newly diagnosed HT patient anthropometry, body composition, insulin, lipid profile, and thyroid function test including TPO.

Case report

A 49-year-old woman, married and with three children, non-smoker, non-alcoholic, and medically free from any chronic diseases was newly diagnosed with HT of unknown cause by the specialized internist endocrinologist. She was medically insured at King Abdullah University Hospital and an assistant professor of clinical nutrition. She sought medical advice after she noticed her body weight started to increase although her usual food intake had not changed, as well as fogginess, constipation, and consistent fatigue. The woman decided to follow a special diet designed by her for six months and not taking any medications.

The patient blood test findings at diagnosis (basal) are shown in Table 1. The insulin and fasting blood glucose (FBG) data were within the normal reference range, 9.97 mIU/L and 5.78 mmol/L respectively. The total cholesterol, low-density lipoprotein cholesterol (LDL), and triglycerides (TG) were elevated above normal, while the high-density lipoprotein cholesterol (HDL) was extremely low. In addition, the thyroid function test and the thyroperoxidase antibodies were tested and the basal results revealed that the patient has a positive TPO (945) and elevated thyroid stimulating hormone (TSH), while the triiodothyronine (T3) and the thyroxine (T4) levels were within the normal reference range (Table 1).

In addition, the patient anthropometric and body composition was analyzed at diagnosis. The woman was obese when diagnosed: her body mass index (BMI) was 30.47 kg/m^2 and her waist to hip ratio (WHR) was 0.9 (Table 1).

Before diagnosis, the patient ate every kind of food and never followed a dietary program. On average, she usually consumed about 2000 Kcal/day. Also, she took nutrition supplements of 1000 mg Omega-3 daily and one-a-day tablets for menopausal woman, which contained multivitamin/multimineral and soybean isoflavonesfrom extract as 60 mg.

Before designing the interventional diet, the woman's energy requirements were calculated by giving 30–35 Kcal/day for each kilogram (kg) of the ideal body weight (IBW) [8]. The estimated energy requirement (EER) ranged between 1770–2065 Kcal/day and it was allocated into 50% for carbohydrates, 15% for protein, and 35% for fat [9]. Thus, the requirements ranged between (221–258 g/day) for carbohydrates, (66.4–77.4 g/day) for protein, and (68.8–80.3g/day) for fat.

The interventional diet was designed to provide a total energy of 1200 Kcal/day, 150 g/day of carbohydrates, 45 g/day of protein and 47 g/day from fat. The patient was advised to take three main meals and two snacks; around 240 Kcal breakfast, 480 Kcal on lunch, 320 Kcal on dinner and around 80 Kcal for each snack. Generally, the meals' calories were translated into food menus and these menus were prepared to be a mixture of Paleo, gluten-free, and vegan diet (modified autoimmune Paleo diet, (MAIPD). In these menus all wheat-based bread and pasta, and all foods containing gluten – such as cereals, baked goods, snack foods, alcoholic beverages – were avoided. All canned and processed foods, caffeine, seaweed, high glycemic index food, soy, beans and legumes, cruciferous vegetables, and nightshades were also avoided. On the other hand, eggs, plain dairy products, meat and fish, fats and oils, nuts and seeds, corn, quinoa, rice, herbs, spices, fruits, and vegetables, except the cruciferous and nightshades, were allowed.

All the blood tests, anthropometric and body composition analysis were repeated every 30 days after starting the dietary intervention for a total of six times throughout the study on days 30, 60, 90, 120, 150, and 180.

Over the dietary intervention, results revealed a positive improvement in her

https://doi.org/10.46389/rjd-2020-1055
© 2020 The Authors

Table 1: Changes of anthropometry, body composition and biochemical parameters over time for a woman with Hashimoto thyroiditis and received medical nutritional therapy.

	Days of dietary plan follow up						
Variable	**Basal**	**30**	**60**	**90**	**120**	**150**	**180**
Age (years)	49.0	49.1	49.2	49.3	49.4	49.5	49.6
Anthropometric measurements							
Weight (Kg)	86.0	83.0	79.50	76.5	74.00	72.0	70.0
Height (Cm)	168.0	168.0	168.0	168.0	168.0	168.0	168.0
BMI (kg/m^2)	30.47	29.41	28.17	27.10	26.22	25.51	24.80
Waist circumference (cm)	106.0	102.0	96.0	91.0	88.0	85.0	83.0
Hip circumference (cm)	118.0	115.0	112.0	109.0	107.0	106.0	105.0
WHR	0.90	0.87	0.86	0.83	0.82	0.80	0.79
Body composition							
Fat mass %	37.5	34.5	31.7	28.8	27.0	26.1	25.3
Fat free mass %	62.5	65.5	68.3	71.2	73.0	73.9	74.7
Total body water %	45.2	47.6	49.3	51.7	52.6	53.8	55.0
Dry lean weight %	13.8	13.5	13.2	13.0	12.8	12.6	12.4
Biochemical measurements							
Insulin (mIU/L)	9.97	9.30	7.94	7.77	7.56	7.50	7.3
Fasting blood glucose (mmol/L)	5.78	5.70	5.66	5.50	5.60	5.44	5.35
Total cholesterol (mmol/L)	8.47	7.10	6.50	5.90	5.24	4.67	4.32
LDL (mmol/L)	6.30	6.15	5.76	4.89	4.63	4.20	3.97
HDL (mmol/L)	0.77	0.85	0.98	1.10	1.24	1.29	1.34
Triglycerides (mmol/L)	2.70	2.51	2.23	2.00	1.88	1.73	1.62
Triiodothyronine T3 (pmol/L)	5.23	4.74	4.30	4.23	4.10	4.38	4.01
Thyroxine T4 (pmol/L)	10.67	11.81	13.20	14.33	14.77	13.16	16.52
Thyroid stimulating hormone TSH (mIU/L)	5.33	4.51	3.30	3.00	2.63	1.36	0.81
TPO (positive)	954	940	865	725	612	543	423

anthropometric and body composition. Insulin and FBG decreased significantly (7.3 mIU/L, 5.35 mmol/L) but remained within the normal range. The patient's total cholesterol, LDL, and TG levels decreased and the HDL level was increased significantly. In addition, the TSH and the TPO levels significantly decreased while the T3 and T4 levels remain within normal reference range (Table 1).

Discussion

It is widely known that thyroid dysfunction affects the patient's physical and mental well-being and decreases the personal health-related quality of life [10]. Results from this prospective, longitudinal case study revealed that the MAIP weight reducing diet was helpful in improving the anthropometric, body composition, and biochemical data of the HT patient. The significant decreases in body weight, BMI, WHR, fat mass, and fasting insulin are associated with minimizing the risk of type 2 diabetes and cardiovascular diseases (CVD). Recently, a study reported that total homocysteine as independent risk factor for CVD is positively associated with BMI, WHR, and fat mass [11]. By the time of dietary intervention, the reductions in anthropometric measures and fat mass were correlated with the reductions in TSH and TPO. These findings are in line with Marzullo et al [12], although other

© 2020 The Authors

studies reported conflicting results regarding the correlations between body weight, BMI, fat mass percentage, and the thyroid function [12–15].

It is more common among hypothyroidism patients to suffer from dyslipidemia [16]. However, the mechanism of how thyroid gland hormones can influence lipid profile is not yet clear [17]. In this case study, total cholesterol, LDL, and TG were significantly reduced and HDL was significantly elevated. These findings assure the effect of the diet not only on anthropometry and body composition but also on lipid profile of hypothyroidism patients. On the contrary, in a dietary interventional randomized controlled study conducted on subclinical hypothyroidism, children who followed a diet consisting of 300 ml of full fat milk and 5 grams butter on each slice of bread daily, green vegetables five times per week and beef three times per week for 6 months did not report significant effects on the participants' lipid profile at the end of the study, though slight, non-significant increases in total cholesterol, LDL and HDL levels and a decrease in TG and total cholesterol/HDL over time were observed in the intervention group [18].

Dietary interventions improve the symptoms of autoimmune thyroid dysfunction and may eliminate the symptoms and the disease. For instance, gluten-free diet with vitamin and minerals supplements was used for a patient diagnosed with HT, celiac disease, and diabetes, to improve his clinical symptoms and laboratory results [19]. Another study compared patients on thyroxin treatment but not adhering to gluten-free diet with patients only following gluten-free diet and found that gluten-free diet patients were able to normalize their TSH levels without taking T4 supplementation [20]. Others suggested the use of the Paleolithic-style diet to prevent HT [21]. Therefore, the improvements in clinical and biochemical prognosis of HT can be explained by the using of the caloric restriction MAIPD to lower the intestinal and the thyroid gland inflammation.

Conclusion

The MAIPD was able to improve the TSH, TPO, anthropometric, body composition, HDL, and non-HDL cholesterol levels. These improvements will help the HT patients to improve their health and quality of life as well as reduce inflammation, thyroxin treatment dose, and risk for chronic diseases associated with hypothyroidism in future.

Financial support

No funds have been received for the present study.

Conflict of interest

The author declares no conflict of interest.

References

1. Vanderpump MP, Tunbridge WM. Epidemiology and prevention of clinical and subclinical hypothyroidism. *Thyroid*. 12:839–847, 2002.
2. Bossowski A, Moniuszko M, Dąbrowska M, et al. Analysis of T regulatory cells in the peripheral blood in children and adolescents with Graves' disease and Hashimoto's thyroiditis. *Endokrynologia Pediatryczna*. 34(1):37–48, 2011.
3. Zaletel, K. Determinants of thyroid autoantibody production in Hashimoto's thyroiditis. *Expert Rev Clin Immunol*. 3: 217–223, 2007.
4. Pearce EN, Farwell AP, Braverman LE. Thyroiditis. *N Engl J Med*. 348 (26): 2646–2655, 2003.
5. Ventura A, Neri E, Ughi C, et al. Gluten-dependent diabetes-related and thyroid-related autoantibodies in patients with celiac disease. *J Pediatr*. 137(2):263–265, 2000.
6. Jiskra J, Limanova Z, Vanickova Z, et al. IgA and IgG antigliadin, IgA anti-tissue transglutaminase and antiendomysial antibodies in patients with autoimmune thyroid diseases and their relationship to thyroidal replacement therapy. *Physiol Res*. 52(1):79–88, 2003.
7. Ballantyne S. *The Paleo Approach: Reverse Autoimmune Disease and Heal Your Body*. Las Vegas: Victory Belt Publishing; 2014.
8. Metropolitan Life Insurance Company. *Metropolitan height and weight tables*. *Stat Bull Metrop Insur Co*. 64:1–9, 1983.
9. Austin GL, Ogden LG, Hill JO. Trends in carbohydrate, fat, and protein intakes and association withenergy intake in normal-weight, overweight, and obese individuals: 1971–2006. *Am J Clin Nutr*. 93:836–843, 2011.
10. Uysal HB, Ayhan M. Autoimmunity affects health-related quality of life in patients with Hashimoto's thyroiditis. *Kaohsiung J Med Sci*. 32: 427–433, 2016.
11. Al-Bayyari N, Hamadneh J, Hailat R, et al. Total homocysteine is positively correlated with body mass index, waist-to-hip ratio, and fat mass among overweight reproductive woman: a cross-sectional study. *Nutr Res*. 48, 9–15, 2017.
12. Marzullo P, Minocci A, Mele C, et al. The relationship between resting energy expenditure and thyroid hormones in

 https://doi.org/10.46389/rjd-2020-1055 © 2020 The Authors

response to short-term weight loss in severe obesity. *PLoS One*. 13:e0205293, 2018.

13. De Pergola G, Ciampolillo A, Paolotti S, et al. Free triiodothyronine and thyroid stimulating hormone are directly associated with waist circumference, independently of insulin resistance, metabolic parameters and blood pressure in overweight and obese woman. *Clin Endocrinol*. 67: 265–269, 2007.
14. Iacobellis G, Ribaudo MC, Zappaterreno A, et al. Relationship of thyroid function with body mass index, leptin, insulin sensitivity and adiponectin in euthyroid obese woman. *Clin Endocrinol*. 62: 487–491, 2005.
15. Manji N, Boelaert K, Sheppard MC, et al. Lack of association between serum TSH or free T4 and body mass index in euthyroid subjects. *Clin Endocrinol*. 64:125–128, 2006.
16. Unal E, Akın A, Yıldırım R, et al. Association of subclinical hypothyroidism with dyslipidemia and increased carotid intima-media thickness in children. *J Clin Res Pediatr Endocrinol*. 9(2):144–149, 2017.
17. Goldberg IJ, Huang LS, Huggins LA, et al. Thyroid hormone reduces cholesterol via a non-LDL receptor-mediated pathway. *Endocrinology*. 153. (11):5143–5149, 2012.
18. Van der Gaag E, Van der Palen J, Schaap P, et al. A lifestyle (dietary) intervention reduces tiredness in children with subclinical hypothyroidism, a randomized controlled trial. *Int J Environ Res Public Health*. 17: 3689, 2020.
19. Schreiber F, Zoib T, Veith M, et al. Atypical celiac disease in a patient with type 1 diabetics mellitus and Hashimoto's thyroiditis. *Deutsche Medizinischen Wochenschriftt*. 136(3): 1–24, 2011. doi:10.1055/s-0030-1269443
20. Virili C, Bassotti G, Santaguida M, et al. Atypical celiac disease as cause of increased need for thyroxine: a systematic study. *J Clin Endocrinol Metab*. 97 (3): E419–E422, 2012. doi:10.1210jc.2011-1851.
21. Kowalski LM, Bujko J. Evaluation of biological and clinical potential of paleolithic diet. *Rocz Panstw Zakl Hig*. 63(1):9–15, 2012.

© 2020 The Authors

https://doi.org/10.46389/rjd-2020-1056
www.rjdnmd.org

Rom J Diabetes Nutr Metab Dis
2020; volume 27, issue 4, pages 386-395

Original Research

Whey protein upregulates muscle insulin receptor tyrosine kinase and is comparable to vildagliptin as insulin-sensitizer

Nahla El-Ashmawy[1], Eman Khedr[1], Hoda El-bahrawy[1], Enas El-Mokadem[2], Mariam Abo-Saif[1]*

[1] Department of Biochemistry, Faculty of Pharmacy, Tanta University, Tanta, Egypt
[2] Outpatient Pharmacy, Chest Department, Meet-Ghamr General Hospital, Meet-Ghamr, Egypt

*Correspondence to: Mariam Abo-Saif, Faculty of Pharmacy, El-Geesh street, Tanta Tani, Tanta, Egypt, 3111.
E-mail: maryem_abosaif@pharm.tanta.edu.eg, Phone: (040) 333-6007

Received: 6 August 2020 / Accepted: 19 November 2020

Abstract

Background and Aims: Whey protein is a natural product with an anti-hyperglycemic effect. This study aimed to evaluate the possible therapeutic effect of different concentration of whey protein compared to vildagliptin on Type 2 diabetes mellitus (T2DM) rat model and to clarify the underlying molecular mechanisms. **Material and Method:** Sixty male Wistar rats were divided into six groups: normal control, diabetes control, vildagliptin treated diabetic group, whey protein 10%, 20%, and 40% treated groups. **Results:** each of vildagliptin and whey protein exhibited anti-hyperglycemic and insulinotropic effect in diabetic rats; however the effects obtained by 40% whey protein was comparable to that of vildagliptin. The biochemical results were supported by the histopathological finding which showed significant increase in the number of β-cells in both vildagliptin and 40% whey protein versus the control group. Interestingly, 40% Whey protein was superior to vildagliptin in increasing the level of muscle insulin receptor tyrosine kinase (IRTK) thereby increasing sensitivity to insulin. **Conclusion:** the anti-hyperglycemic effect of whey protein was concentration-dependent and mediated by increasing intestinal incretin hormones, muscle (IRTK), and number of β-cells. 40% whey protein was comparable to vildagliptin as antihyperglycemic drug proved to have an insulin-sensitizing effect. It could be used safely as a substitute for diabetic patients.

Keywords: Incretin, incretin hormones, muscle; Type 2 diabetes mellitus.

Background and Aims

Type 2 diabetes mellitus (T2DM) is considered one of the most popular metabolic disorders. T2DM can contribute to early mortality and micro- and macro-vascular complications, including stroke, myocardial infarction, and loss of vision [1].

The decline of β-cell function in T2DM has been associated with the pathophysiological changes of impaired action of incretin hormones, which are secreted from the intestine in response to glucose intake [2]. Incretin hormones include glucose-dependent insulinotropic polypeptide (GIP) and glucagon like peptide-1 (GLP-1) which are responsible for stimulation of insulin secretion after glucose ingestion andinhibits glucagon release [3].

Dipeptidyl peptidase-4 (DPP4) inhibitors are an oral hypoglycemic agent. DPP4 inhibitors stimulate insulin secretion and inhibit glucagon release by elevating endogenous GLP-1 and GIP levels without risk of hypoglycemia [4]. Besides, DPP4 inhibitors improve pancreatic β-cell function by stimulating the proliferation of β-cells and inhibition of apoptosis [5]. Currently, many DPP4 inhibitors are approved for the treatment of T2DM, including saxagliptin, sitagliptin, and vildagliptin [6].

Vildagliptin elevates GIP and GLP-1 by inhibiting DPP-4, leading to improve in both fasting blood glucose (FBG) and postprandial

© 2020 The Authors. Romanian Journal of Diabetes, Nutrition and Metabolic Diseases published by Sanatatea Press Group on behalf of the Romanian Society of Diabetes Nutrition and Metabolic Diseases. This is an open access article under the terms of the Creative Commons Attribution License (CC-BY-NC-ND 3.0).

blood glucose (PBG) [4]. Vildagliptin increases insulin secretion and pancreatic insulin stores. Moreover, vildagliptin can increase β-cell mass and ratio [7]. On the other hand, vildagliptin has some adverse effects, including headache, cough, nasopharyngitis, fever, constipation, and gastrointestinal discomfort [8, 9].

Whey protein has insulinogenic properties as it contains a high level of branched-chain amino acids especially leucine, which are associated with the glucoregulatory effect [10]. This insulinotropic effect of whey is due to the direct effect of amino acids on β-cells to secrete insulin and stimulate incretin, as it inhibits the action of DPP-4, so reducing PBG [11]. Also, whey protein helps to improve inflammation and oxidative stress, which play a critical role in developing diabetes complications [12].

The insulin receptors contain a tyrosine kinase enzyme. Binding of insulin to the α subunit of the receptor activates the β subunit, which leads to autophosphorylation and activation of tyrosine kinase [13]. Insulin promotes the uptake of blood glucose into the skeletal muscle after binding with insulin receptor tyrosine kinase (IRTK). Skeletal muscle is responsible for 85% of insulin-enhanced uptake of glucose from the blood and it is the principal tissue responsible for reducing blood glucose level [14].

The current study was conducted to highlight the effect of different concentrations of whey protein (10%, 20%, and 40%) as an antidiabetic agent compared to vildagliptin against high-fat diet and streptozotocin (HFD/STZ) induced T2DM in rats and to clarify the possible molecular mechanisms underlying whey protein action.

Material and Method

Animal model

Sixty male Wistar rats weighing 90–110 g at approximately five weeks of age were obtained from the National Research Center, Giza, Egypt. The rats were weighed and housed in aluminum cages for two weeks under identical environmental conditions for adaptation and allowed free access to normal chow diet and water.

Experimental design

The study was performed under the guidelines for the care and use of laboratory animals and approved by the Research Ethics Committee of Faculty of Pharmacy, Tanta University, Egypt. After the acclimatization period, rats were weighed and randomly divided into two major groups: normal control group (n=10), and diabetic (n=50). The untreated normal control group was maintained on the control diet and water ad libitum and received a single i.p. dose of the formulation containing the vehicle (0.1 M sodium citrate buffer 0.25 mL/kg). The diabetic rats received HFD for two weeks and then 35 mg/kg STZ injection to develop T2DM model (HFD/STZ). The STZ injection is a single i.p. dose of STZ (Sigma Aldrich®, USA) dissolved in 0.1 M sodium citrate buffer, pH 4.5. That model aimed to develop T2DM pathology progression, and insulin resistance or hypoinsulinemia, in a condensed timeline [15]. The composition of the control diet and HFD are shown in Table 1.

After three days, STZ induced β-cell-toxicity and induces experimental diabetes. STZ is capable of producing fatal hypoglycemia as a result of massive pancreatic insulin release so, the rats were maintained on 5% glucose solution via their drinkers for the 24 hour following STZ administration to prevent hypoglycemia [16].

Blood glucose was tested using a blood glucose meter (Accu-Chek Performa; Roche Diagnostics®, USA). All rats with blood glucose concentrations greater than 16.7 mmol/l were considered to be diabetic and were selected for further research according to the American Diabetes Association [17]. Diabetic rats were further randomly divided into five subgroups (n=10); Group 1: untreated diabetic group received 1 mL of the formulation containing the vehicle. Group 2: vildagliptin (Novartis International AG®, Switzerland) treated group received 1 mL of the formulation vildagliptin (10 mg/kg) once a day via oral gavage for four consecutive weeks [18].

Groups 3, 4 and 5: whey protein 10%, 20% and 40% treated groups respectively received 1mL of the formulation containing whey protein (1 mL/100 g) once a day via oral gavage for four consecutive weeks. Whey protein was obtained

© 2020 The Authors

Table 1: The composition of the control diet and high fat diet (HFD)

Diet components	Control diet	HFD
Energy per day (Kcal/g)	3	4
Calorie percent:		
protein	22	20 (casein)
Fat	12	45 (lard)
Carbohydrate	66	35 (corn starch, maltodextrin, and sucrose)

from Optimum Company®, USA and was dissolved in distilled water to prepare the different concentrations (10%, 20%, and 40%).

Specimen collection

At the end of the experiment, rats were weighed and the blood was withdrawn from the eye veins after overnight food deprivation for determination of FBG. The remaining blood was immediately centrifuged for 15 minute at 3000 rpm and serum was stored at -80°C for the determination of fasting insulin. The fasted rats were allowed to eat and the PBG was determined after two hour. Rats were then sacrificed and their pancreas, small intestines, and skeletal muscles were dissected. The fresh pancreas was washed twice in ice-cold saline dried on clean paper towels and kept in formalin solution for histopathological examination. The small intestine was kept frozen at -80°C till the determination of the concentration of GIP and GLP-1. Skeletal muscles were kept frozen at -80°C till the measurement of IRTK concentration.

Determination of blood glucose concentration

Accu-Chek advantage capillary glucose meter (Roche Diagnostics®, Germany) was used for the immediate determination of FBG and PBG in whole blood using the noble metal electrode strip.

Determination of serum insulin level

Serum insulin concentration was measured by rat insulin ELISA kit, obtained from MLbio Biotechnology®, China. The concentration of insulin was determined according to manufacturer procedure and expressed as μIU/mL. Insulin resistance was determined by homeostasis model assessment-insulin resistance (HOMA-IR) index described by Matthews et al [19], which was calculated as follows:

$$\text{HOMA-IR} = \frac{\text{FBG (mg/dL) X fasting insulin (μU/mL)}}{405}$$

Determination of glucose dependent insulinotropic polypeptide (GIP) in the small intestine

It was determined in small intestine tissue by rat GIP ELISA kit obtained from MLbio Biotechnology®, China. The concentration of GIP was determined according to the manufacturer procedure and was expressed as pg\g tissue.

Determination of glucagon like peptide-1 (GLP-1) in the small intestine

It was determined in the rat's small intestine tissue by rat GLP-1 ELISA kit obtained from MLbio Biotechnology®, China. The concentration of GLP-1 was determined according to the manufacturer procedure and was expressed as pg\g tissue.

Determination of insulin receptor tyrosine kinase (IRTK) in skeletal muscles

Rat IRTK ELISA kit obtained from MLbio Biotechnology®, China was used to measure IRTK in rat skeletal muscles. The concentration of IRTK was determined according to the manufacturer procedure and was expressed as pg\g tissue.

Histopathological examination

Transverse pancreas sections were carefully embedded in molten paraffin and kept

https://doi.org/10.46389/rjd-2020-1056
© 2020 The Authors

under freezing plates to allow the paraffin to solidify. Cross sections (5 µm thick) of the fixed tissues were cut. These sections were stained with hematoxylin and eosin (H&E) stain for general histopathological examination. Images were viewed and recorded using Olympus microscope equipped with spot digital camera using computer program MATLAB software by abroad certified pathologist in Pathology Department, Faculty of Veterinary Medicine, Mansoura University, Mansoura, Egypt. The investigator performing the histological evaluation was blind to biochemical results and treatment allocation.

Statistical analysis

Analysis of data was performed with the statistical package for social science (SPSS) software version 22 [20]. Data are presented as % change and mean ± SEM. Statistical comparison among groups was performed using one-way analysis of variance (ANOVA) using Fisher's least-significant differences (LSD) method for comparison between two groups. Statistical significance was set at $p< 0.05$.

Results

Effect of whey protein and vildagliptin on blood glucose, insulin level, and HOMA-IR

Diabetic control rats showed a significant increase ($p<0.05$) in the level of FBG and PBG (189.3±1.78 mg/dL and 335.2±17.9 mg/dL, respectively) versus the normal control group (86±1.86 mg/dL and 120±3 mg/dL, respectively). Treatment with vildagliptin significantly decreased ($p<0.05$) FBG and PBG level (92.8±1.5 mg/dL and 128.1±21 mg/dL, respectively) versus the diabetic control group. Treatment with 10%, 20% and 40% whey protein significantly decreased ($p<0.05$) FBG level (158.8±1.6 mg/dL, 126.4±1.9 mg/dL, and 79.6±1.27mg/dL, respectively) compared to the diabetic control group. Besides, treatment with 10%, 20% and 40% whey protein significantly decreased ($p<0.05$) PBG level (184.7±1.82 mg/dL, 151.6±2.71 mg/dL, and 122.4±2.43 mg/dL, respectively) compared to the diabetic control group (Fig. 1A & B).

Injection with STZ caused a significant decrease ($p<0.05$) in fasting blood insulin level in the diabetic group (7.39±0.02µIU/mL) versus the normal control group (8.81±0.06µIU/mL). Treatment with vildagliptin significantly increased ($p<0.05$) the level of fasting insulin (8.83±0.05µIU/mL) versus the diabetic control group. Treatment with 10%, 20% and 40% whey protein significantly increased ($p<0.05$) the level of fasting insulin (7.83±0.05µIU/mL, 8.36±0.03µIU/mL, and 9.37±0.03µIU/mL, respectively) versus the diabetic control group (Fig. 1C).

HOMA-IR values were calculated for the estimation of insulin resistance in the different groups. Injection with STZ caused a significant increase ($p<0.05$) in the HOMA-IR value of the diabetic group (3.46±0.1)versus the normal control group (1.87±0.03). Treatment with vildagliptin significantly decreased ($p<0.05$) HOMA-IR value (2.01±0.1) compared to the diabetic control group. Treatment with 10%, 20% and 40% whey protein significantly decreased ($p<0.05$) HOMA-IR value (3.071±0.03, 2.608±0.04, and 1.842±0.03, respectively) compared to the diabetic control group (Fig. 1D). Interestingly, 40% whey protein group showed a reduction in the blood glucose and HOMA-IR value to a level comparable to that of the normal control group (Fig. 1).

Effect of whey protein and vildagliptin on intestinal GIP and GLP-1 concentrations

The diabetic control group showed a significant decrease ($p<0.05$) in GIP concentration (3.64±0.12 ng/g) versus the normal control group (6.16±0.12 ng/g). Treatment with vildagliptin showed a significant increase ($p<0.05$) in GIP concentration (18.8±0.13 ng/g) versus the diabetic control group. Treatment with 10%, 20%, and 40% whey protein significantly increased ($p<0.05$) GIP concentration (7.71±0.18 ng/g, 12.12±0.32 ng/g, and 14.82±0.24 ng/g, respectively) versus the diabetic control group (Fig. 2A).

The diabetic control group showed a significant decrease ($p<0.05$) in GLP-1

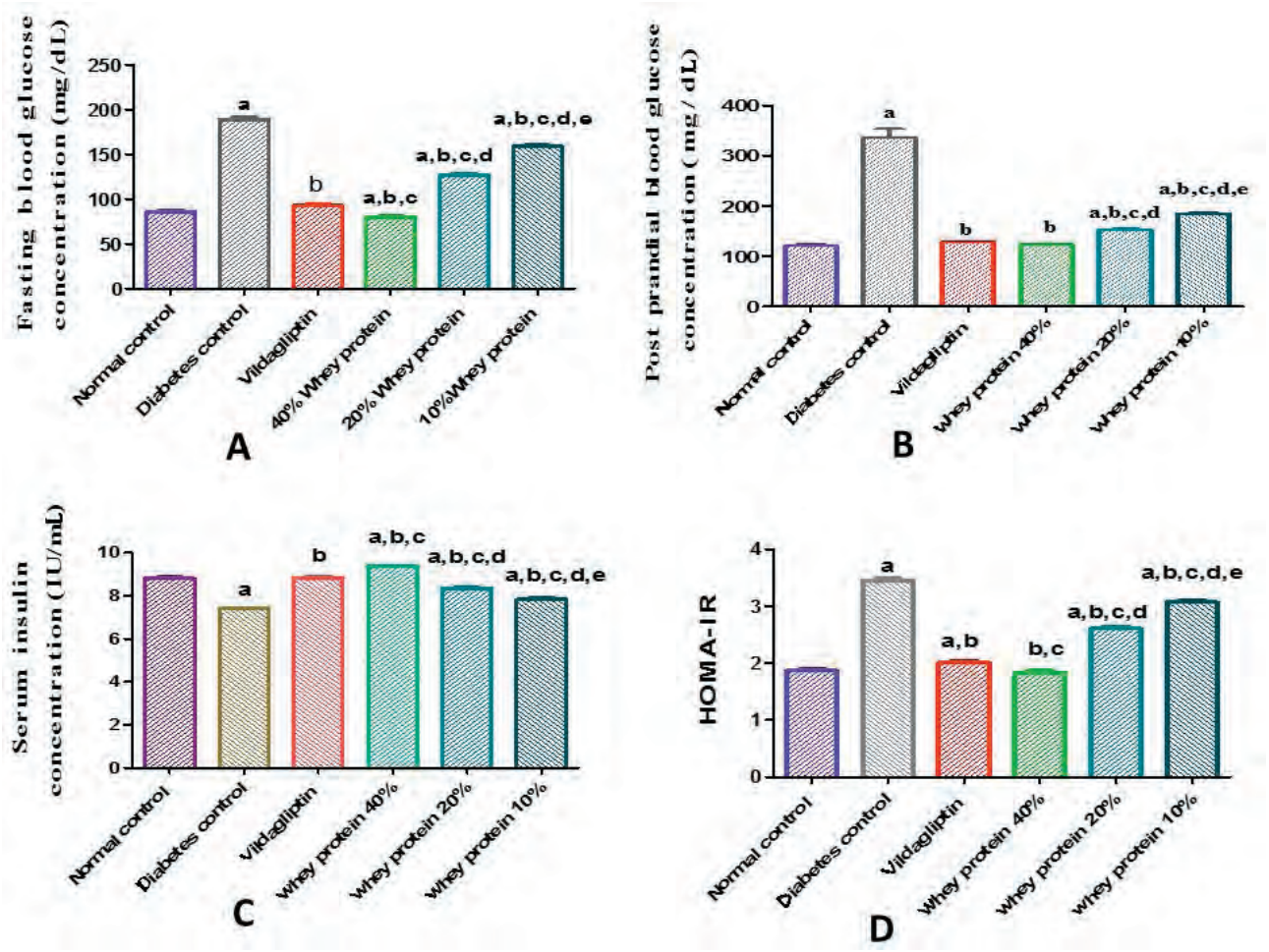


Figure 1: (A) Fasting blood glucose level in rat groups. (B) Postprandial blood glucose level in rat groups. (C) Fasting insulin level in the serum of rat groups. (D) HOMA-IR in rats groups. Data are presented as mean ± SEM (n= 10/group), significance was set at $p<0.05$; a: Significant versus normal control group, b: Significant versus diabetic control group, c: Significant versus vildagliptin group, d: Significant versus 40% whey protein group, e: Significant versus 20% whey protein group.

concentration (9.22±0.37 pg/g) versus the normal control group (13.41±0.22 pg/g). Treatment with vildagliptin showed a significant increase ($p<0.05$) in GLP-1 concentration (33.38±0.64 pg/g) versus the diabetic control group. Treatment with 10%, 20% and 40% whey protein significantly increased ($p<0.05$)GLP-1 concentration (16.95±0.6 pg/g, 24.24±0.54 pg/g, and 28.55±0.29 pg/g, respectively) versus the diabetic control group (Fig. 2B).

Effect of whey protein and vildagliptin on IRTK in skeletal muscles

Injection with STZ caused a significant decrease ($p<0.05$) in IRTK concentration in skeletal muscles isolated from the diabetic control group (1340±92.74 pg/g) versus the normal control group (2583±85 pg/g). Treatment with vildagliptin showed a non-significant increase in IRTK concentration (1488±101 pg/g) versus the diabetic control group. On the other hand, treatment with 10%, 20% and 40% whey protein significantly ($p<0.05$) increased IRTK concentration (2130±181.2 pg/g, 3168±90.45 pg/g, and 4273±82.28 pg/g, respectively) versus the diabetic control group (Fig. 2C).

Effect of whey protein and vildagliptin on the histopathology of the pancreas

By using MATLAB software the number of β-cells per islet can be determined. Injection with STZ caused a significant decrease ($p<0.05$) in the number of β-cells (27.33±2.23) versus the normal control rats (172.2± 9.86) (Fig. 2D). The sections

https://doi.org/10.46389/rjd-2020-1056
© 2020 The Authors

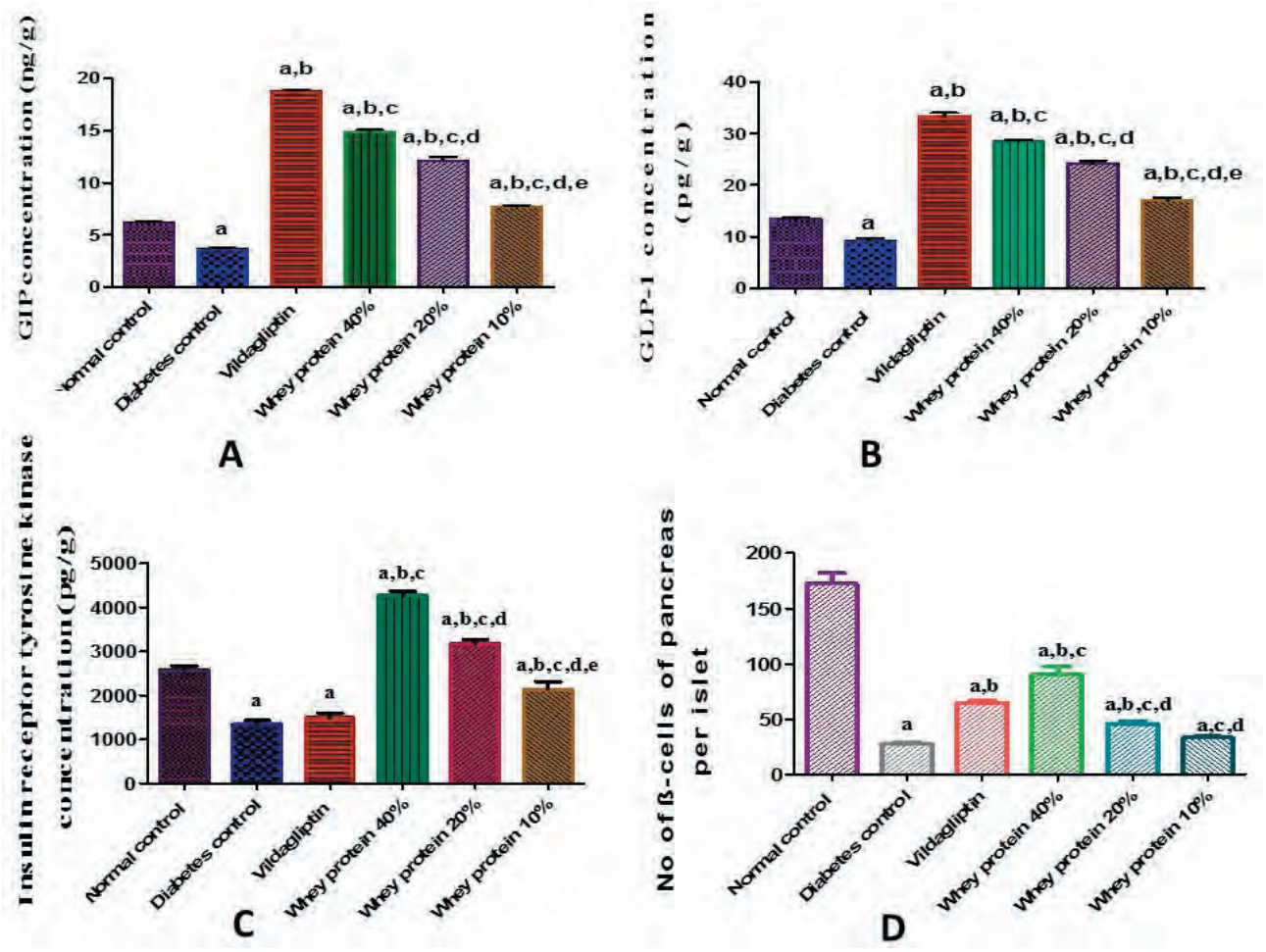


Figure 2: (A) Concentration of intestinal GIP in rats groups. (B) Concentration of Intestinal GLP-1 in rats groups. (C) Concentration of insulin receptor tyrosine kinase in skeletal muscles of rats groups. (D) Number of β-cells of the pancreases per islet in rats groups. Data are presented as mean ± SEM (n= 10/group), significance was set at $p<0.05$; a: Significant versus normal control group, b: Significant versus diabetic control group, c: Significant versus vildagliptin group, d: Significant versus 40% whey protein group, e: Significant versus 20% whey protein group.

from the pancreas of the normal control group showed normal islets of Langerhans containing normal population of α and β-cells, normal blood capillaries embedded in exocrine portion with normal intercalated duct (Fig. 3A). The sections from the pancreas of the diabetes control group showed signs of inflammation indicated by the deposition of pale eosinophilic amyloid substance in islets of Langerhans with degeneration and necrosis of β-cells, normal exocrine portion of the pancreas, and normal intercalated duct lined by simple cuboidal epithelium (Fig. 3B).

Treatment with vildagliptin or 40% whey protein showed a significant increase ($p<0.05$) in the number of β-cells as shown by the large proportion and number of islet cells and restoring the histological architecture of islets of Langerhans (Fig. 3C and D). Treatment with 20% whey protein showed significant increase ($p<0.05$) in the number of β-cells and moderate restoring of the histological architecture of islets of Langerhans with mild deposition amyloid substance (Fig. 2D and Fig. 3E).

On the other hand, the section from the pancreas of 10% whey protein treated group showed the presence of amyloid substance with mild restoration of β-cells of islets of Langerhans which is non-significant when compared to the diabetic group (Fig. 2D and Fig. 3F).

Discussion

The prevalence of diabetes is increasing around the world, affecting approximately 424.9×10^6 people in 2017 and expected to increase

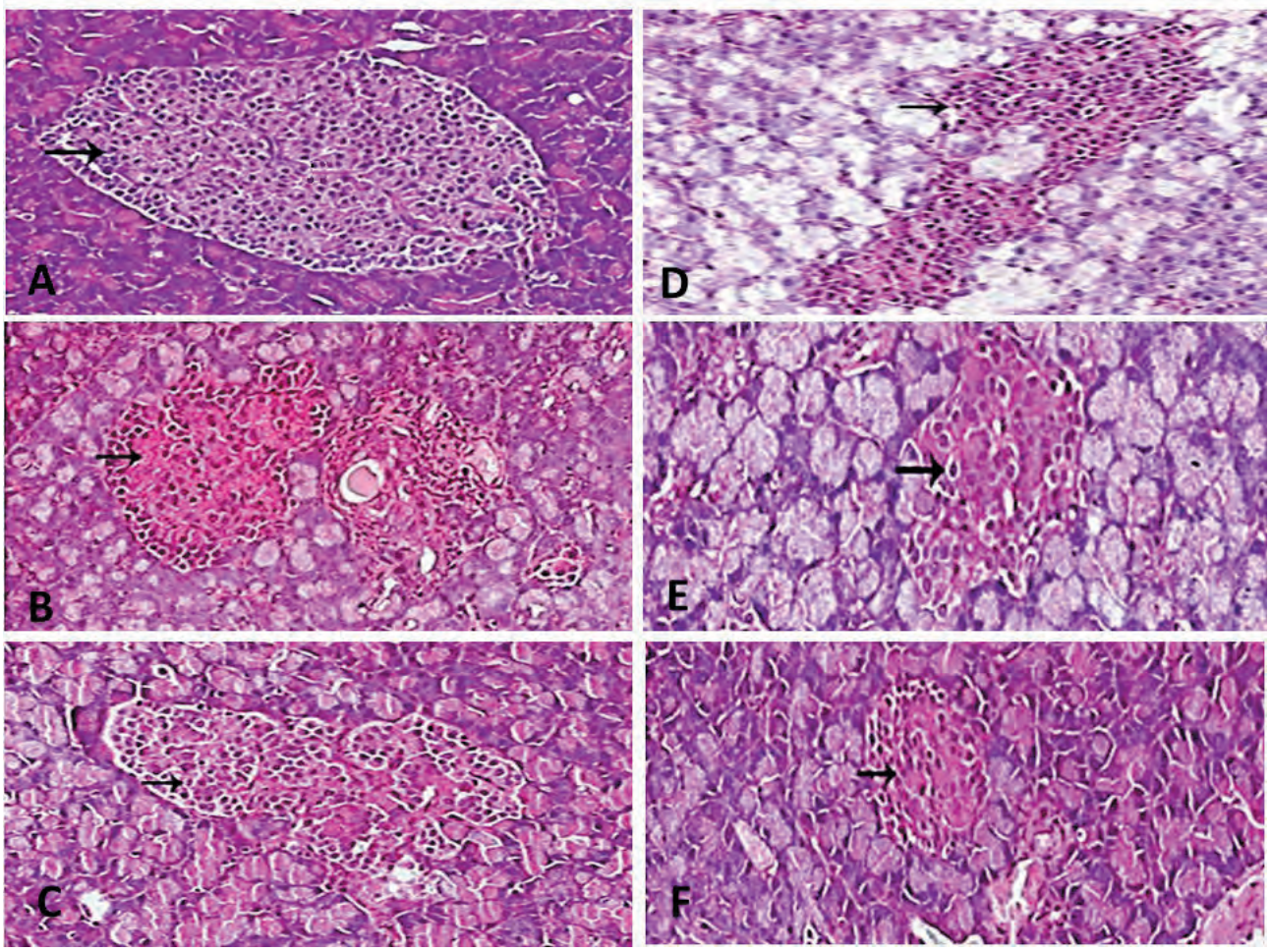


Figure 3: Sections from the pancreas (H&E, 400x) of (A) normal control group showing normal islets of Langerhans containing a population of α and β-cells (arrow) and were all present in their normal proportions. (B) diabetic control group showing deposition of pale eosinophilic amyloid substance in islets of Langerhans (arrow) with degeneration and necrosis of β-cells. The dimension and the number of cells in each islet of Langerhans were also reduced. (C) vildagliptin treated group showing an increase in the number of β-cells (arrow) and restoring the histological architecture of islets of Langerhans, The number and size of islet of Langerhans were increased as compared to the diabetic control group. The number of cells in each islet was also increased. (D) 40% whey protein treated group showing an increase in the number of β-cells (arrow) and restoring the histological architecture of islets of Langerhans. The islets are present with a large proportion and number of islet cells. (E) 20% whey protein treated group showing a mild increase in the number of β-cells (arrow) and a mild restoring the histological architecture of islets of Langerhans with mild deposition amyloid substance. (F) 10% whey protein treated group showing a small increase in the number of β-cells (arrow) with necrosis of β-cells and a mild deposition amyloid substance.

to 628.6 x10^6 in 2045 [21]. Nowadays, alternative and complementary medicine provide us with many natural products, including whey protein which approved its therapeutic value in reducing blood glucose levels in diabetes [12]. This study was conducted to compare the therapeutic effects of different concentrations of whey protein (10–20%, and 40%) with vildagliptin (DPP4 inhibitor) in T2DM rat model and to clarify the molecular mechanisms underlying whey protein action.

Experimental T2DM was induced in the present study in male Wistar rats by (HFD/STZ) and the diabetes control group showed a significant elevation of both FBG and PBG versus normal controls. Diabetes induction was also evidenced by the histopathological changes of the pancreas which showed deposition of pale eosinophilic amyloid substance and vacuolizations in islets of Langerhans with degeneration and necrosis of β-cells. The number of β-cells per islets in the diabetes control group was significantly lower than that of the normal control group.

Our findings demonstrate that treatment with vildagliptin or whey protein 10%-20%-40%)

https://doi.org/10.46389/rjd-2020-1056
© 2020 The Authors

reversed the effect of STZ significantly on blood glucose levels in diabetic control rats. Our study showed that treatment with whey protein significantly decreased FBG and PBG in a concentration-dependent manner and 40% whey protein decreased the level of blood glucose to a level comparable or lower than that of vildagliptin treated group. Our results were in agreement with King et al [11] who reported that a small dose of whey protein before meals improves PBG in men with T2DM.

These results were matched with our observed histopathological findings as the section of the pancreas of vildagliptin or 40% whey protein treated group showed a significant increase in the number of β-cells as shown by the large proportion and number of islet cells and restoring the histological architecture of islets of Langerhans. Treatment with 20% showed an increase in the number of β-cells and moderate restoring of the histological architecture of islets of Langerhans with mild deposition amyloid substance. While the pancreas of 10% whey protein group showed the presence of amyloid substance with mild restoration of β-cells of islets of Langerhans which is non-significant when compared to the diabetic group.

In our study, we determined the number of β-cells per islets by MATLAB software. Herein, treatment with vildagliptin or whey protein (20%-40%) showed a significant increase in the number of β-cells versus the diabetic control group. Our study showed that treatment with whey protein (10%-20%-40%) increased the number of β-cells in a concentration-dependent manner and 40% whey protein increased the number of β-cells to a level significantly higher than that of vildagliptin treated group. Our results were in agreement with Argun-Kurum et al [22] who showed that vildagliptin promotes proliferation of islet cell and rearranges the morphology of islet in diabetic rats.

As shown by our results, injection with STZ caused a significant decrease in fasting blood insulin levels in the diabetic group versus the normal control group. Treatment with vildagliptin or whey protein (10%-20%-40%) significantly reversed the effect of STZ on insulin secretion. Interestingly our results showed that treatment with whey protein significantly increased insulin secretion in a concentration-dependent manner and 40% whey protein increased the level of blood insulin to a level significantly higher than that of the vildagliptin treated group. This goes with the results reported by Wildova et al [23] who found that oral administration of whey protein increases the serum C-peptide in diabetic patients.

For the estimation of insulin resistance, we calculated HOMA-IR values in the different groups. Our results showed that injection with STZ caused a significant increase in the HOMA-IR value of the diabetic group versus the normal control group while treatment with vildagliptin or whey protein (10%-20%-40%) significantly decreased HOMA-IR value compared to the diabetic control group. Our results were in line with Tong et al [24] who reported that the supplementation of 15% whey protein significantly decreases HOMA-IR in non-obese IR rats.

The incretin (GIP and GLP-1) increase insulin secretion after oral ingestion of carbohydrate [3]. In the present study, the diabetic control group showed a significant decrease in GIP and GLP-1 concentration versus the normal control group. Furthermore, treatment with vildagliptin or whey protein (10%-20%-40%) showed a significant increase in GIP and GLP-1 concentration versus the diabetic control group. Moreover, treatment with whey protein significantly increased GIP and GLP-1 levels in a concentration-dependent manner. In support of our finding, Giezenaar et al [25] demonstrated that whey protein causes an increase in concentrations of insulin, GIP and GLP-1 in older men and women.

One of the mechanisms of insulin to decrease blood glucose is to induce glucose uptake by the skeletal muscles via activation of IRTK [26]. In the current study, injection with STZ caused a significant decrease in IRTK concentration in skeletal muscles isolated from the diabetic control group versus the normal control group. Our study showed for the first time that, treatment with whey protein (10%-20%-40%) showed a significant increase in IRTK concentration versus the diabetic control group and vildagliptin treated group. Besides, the increment in

© 2020 The Authors

skeletal muscle content of IRTK due to whey protein treatment is concentration-dependent.

Conclusion

The insulinotropic and anti-hyperglycemic effect of whey protein in T2DM rats is concentration-dependent which could be mediated by increasing each of intestinal concentration of incretin hormones (GIP and GLP-1), skeletal muscle content of IRTK, and number of β-cells of the pancreas. Our results showed that 40% whey protein was comparable to vildagliptin in lowering blood glucose level and HOMA-IR value and increasing insulin secretion. However, 40% whey protein was superior to vildagliptin in increasing the level of muscle IRTK thereby increasing muscle sensitivity to insulin. Being a natural product, 40% whey protein could be used safely as a substitute for synthetic drugs for the treatment of T2DM patients. Future clinical studies are recommended to investigate the beneficial therapeutic efficacy of 40% whey protein over vildagliptin.

Acknowledgments

Authors gratefully acknowledge Dr. Mohamed Fawzy, a pathologist in Pathology Department, Faculty of Veterinary Medicine, Mansoura University, Mansoura, Egypt, for conducting and interpreting the histopathological examination.

Conflict of Interest

The authors declare no conflict of interest.

References

1. Roglic G. WHO Global report on diabetes: A summary. *Int J Noncommunicable Dis*. 1: 3, 2016.
2. Nauck MA, Meier JJ. The incretin effect in healthy individuals and those with type 2 diabetes: physiology, pathophysiology, and response to therapeutic interventions. *Lancet Diabetes Endocrinol*. 4: 525–536, 2016.
3. Holst JJ. The incretin system in healthy humans: the role of GIP and GLP-1. *Metabolism*. 96: 46–55, 2019.
4. Foley JE. Insights Into GLP-1 and GIP Actions Emerging From Vildagliptin Mechanism Studies in Man. *Front Endocrinol (Lausanne)*. 10, 2019.
5. Kawamori D, Shirakawa J, Liew CW et al. GLP-1 signalling compensates for impaired insulin signalling in regulating beta cell proliferation in βIRKO mice. *Diabetologia*. 60: 1442–1453, 2017.
6. Gallwitz B. Clinical use of DPP-4 inhibitors. *Front Endocrinol (Lausanne)*. 10: 1–10, 2019. https://doi.org/10.3389/fendo.2019.00389.
7. Xie C, Wang X, Jones KL et al. Role of endogenous glucagon-like peptide-1 enhanced by vildagliptin in the glycaemic and energy expenditure responses to intraduodenal fat infusion in type 2 diabetes. *Diabetes, Obes Metab*. 22: 383–392, 2020.
8. Deacon CF, Lebovitz HE. Comparative review of dipeptidyl peptidase-4 inhibitors and sulphonylureas. *Diabetes, Obes Metab*. 18: 333–347, 2016.
9. Zhou X, Ding L, Liu J, Su L, Dong J, Liao L. Efficacy and short-term side effects of sitagliptin, vildagliptin and saxagliptin in Chinese diabetes: a randomized clinical trial. *Endocr Connect*. 8: 318–325, 2019.
10. Derosa G, D'Angelo A, Maffioli P. Change of some oxidative stress parameters after supplementation with whey protein isolate in patients with type 2 diabetes. *Nutrition*. 73: 110700, 2020.
11. King DG, Walker M, Campbell MD, Breen L, Stevenson EJ, West DJ. A small dose of whey protein co-ingested with mixed-macronutrient breakfast and lunch meals improves postprandial glycemia and suppresses appetite in men with type 2 diabetes: a randomized controlled trial. *Am J Clin Nutr*. 107: 550–557, 2018.
12. Jakubowicz D, Wainstein J, Landau Z et al. High-energy breakfast based on whey protein reduces body weight, postprandial glycemia and hba1c in type 2 diabetes. *J Nutr Biochem*. 49: 1–7, 2017.
13. Bergeron JJM, Di Guglielmo GM, Dahan S, Dominguez M, Posner BI. Spatial and temporal regulation of receptor tyrosine kinase activation and intracellular signal transduction. *Annu Rev Biochem*. 85: 573–597, 2016.
14. Najjar SM, Perdomo G. Hepatic insulin clearance: mechanism and physiology. *Physiology*. 34: 198–215, 2019.
15. Wang Y, Campbell T, Perry B, Beaurepaire C, Qin L. Hypoglycemic and insulin-sensitizing effects of berberine in high-fat diet-and streptozotocin-induced diabetic rats. *Metabolism*. 60: 298–305, 2011.
16. Babu PS, Srinivasan K. Influence of dietary capsaicin and onion on the metabolic abnormalities associated with streptozotocin induced diabetes mellitus. *Mol Cell Biochem*. 175: 49–57, 1997.
17. Peterson RG, Jackson C V, Zimmerman K, de Winter W, Huebert N, Hansen MK. Characterization of the ZDSD rat: a translational model for the study of metabolic syndrome and type 2 diabetes. *J Diabetes Res*. 2015, 2015.
18. Shishavan MH, Henning RH, Van Buiten A, Goris M, Deelman LE, Buikema H. Metformin improves endothelial function and reduces blood pressure in diabetic spontaneously hypertensive rats independent from glycemia control: comparison to vildagliptin. *Sci Rep*. 7: 1–12, 2017.
19. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia*. 28: 412–419, 1985.

 https://doi.org/10.46389/rjd-2020-1056 © 2020 The Authors

20. Field A. Discovering statistics using IBM SPSS statistics. *Sage*, 2013.
21. Cho NH, Shaw JE, Karuranga S et al. IDF Diabetes Atlas: Global estimates of diabetes prevalence for 2017 and projections for 2045. *Diabetes Res Clin Pract*. 138: 271–281, 2018.
22. Argun-Kurum G, Kaya-Dagistanli F, Ozturk M. DPP4 inhibitor induces beta cell regeneration and DDR-1 protein expression as an endocrine progenitor cell marker in neonatal STZ-diabetic rats. *Pharmacol Reports*. 71: 721–731, 2019.
23. Wildova E, Kraml P, Potočková J, Dlouhý P, Anděl M. The Assessment of the Serum C-Peptide and Plasma Glucose Levels by Orally Administered Whey Proteins in Type 2 Diabetes Mellitus. *Physiol Res*. 66: 993–999, 2017.
24. Tong X, Li W, Xu J-Y, Han S, Qin L-Q. Effects of whey protein and leucine supplementation on insulin resistance in non-obese insulin-resistant model rats. *Nutrition*. 30: 1076–1080, 2014.
25. Giezenaar C, Trahair LG, Luscombe-Marsh ND et al. Effects of randomized whey-protein loads on energy intake, appetite, gastric emptying, and plasma gut-hormone concentrations in older men and women. *Am J Clin Nutr*. 106: 865–877, 2017.
26. De Meyts P. Insulin/receptor binding: the last piece of the puzzle? What recent progress on the structure of the insulin/receptor complex tells us (or not) about negative cooperativity and activation. *Bioessays*. 37: 389–397, 2015.

© 2020 The Authors

https://doi.org/10.46389/rjd-2020-1057
www.rjdnmd.org

Rom J Diabetes Nutr Metab Dis
2020; volume 27, issue 4, pages 396-402

Original Research

Prescribing pattern of dipeptidyl peptidase 4 inhibitors and level of HBA1C target achievements among outpatients with type 2 diabetes mellitus in a Malaysian university teaching hospital

Mohamed Hassan Elnaem[1,2]*, Mohamad Hafiz Hakeem Shamsuri[1], Nur Athirah Mohamad Aziz[1], Nurul Iman Alias[1], Rauha Farhana Hafizi[1], Nur Faezah Latif[1], Arina Norhazwani Rashid[1], Mohd Faris Aiman Mohamad Jalil[1], Mery Wei Ying Hu[3]

[1] Department of Pharmacy Practice, Faculty of Pharmacy, International Islamic University Malaysia, Kuantan, Pahang, Malaysia
[2] Quality Use of Medicines Research Group, Faculty of Pharmacy, International Islamic University Malaysia, Kuantan, Pahang, Malaysia
[3] Department of Pharmacy, Sultan Ahmad Shah Medical Centre@ IIUM, Kuantan, Pahang, Malaysia

*Correspondence to: Mohamed Hassan Elnaem, PharmD, PhD, Department of Pharmacy Practice, Faculty of Pharmacy, International Islamic University Malaysia, 25200 Kuantan, Pahang, Malaysia. E-mail: drmelnaem@gmail.com, drmelnaem@iium.edu.my. Phone: 0060193944726, 00695714600

Received: 25 March 2020 / Accepted: 20 June 2020

Abstract

Background and Aims: Oral antidiabetic drugs, including Dipeptidyl Peptidase 4 Inhibitors (DPP4i), are the mainstay for therapeutic management of type 2 diabetes mellitus (T2DM). We aimed to describe the prescribing pattern of DPP4i agents and to assess the achievement of the target HbA1c levels among current DPP4i users at a Malaysian University teaching hospital. **Material and Method:** A retrospective cross-sectional study was conducted in the outpatient diabetes clinic of a university teaching hospital in Pahang, Malaysia. The data included adults with T2DM who received DPP4i prescriptions at least three months before December 2019 and reported data of HbA1c after at least three months from the index date. Evaluation of the DPP4i prescribing patterns referred to the national clinical practice guidelines. **Results:** 140 cases were included. Sitagliptin 50 mg is the most commonly prescribed DPP4i regimen (72.1%), and its combination therapy with metformin contributed to 67.1% of the total DPP4i prescriptions. About 35% of patients achieved their target HbA1c levels after at least three months on DPP-4 inhibitors therapy. There was no significant association between the type of DPP4i and the target HbA1c achievement (p=0.205). **Conclusions:** DPP4i medications were more prescribed as combination therapy with metformin, compared to monotherapy except for vildagliptin. Gliclazide was the most common co-prescribed OAD with vildagliptin. Barriers to achieving optimal glycemic control for patients on OAD, particularly DPP4i, need further investigations.

Keywords: Dipeptidyl Peptidase 4 Inhibitors, glycemic control, Malaysia, Oral antidiabetic drugs, type 2 diabetes mellitus.

Background and Aims

Diabetes mellitus remains one of the major concerns in Malaysia with a reported prevalence of 17.5% in 2015, the majority of them are type 2 diabetes mellitus (T2DM) cases [1]. The pharmacotherapy of T2DM is directed towards alleviating the symptoms, achieving the target glycemic levels of patients, and preventing complications [2]. One of the common markers used in monitoring the glycemic level is Hemoglobin A1c (HbA1c), which gives a long term profile of diabetes control [3]. Oral antidiabetic drugs (OAD), including Dipeptidyl Peptidase 4 Inhibitors (DPP4i), are the mainstay for the therapeutic management of patients with T2DM. Through their impact

© 2020 The Authors. Romanian Journal of Diabetes, Nutrition and Metabolic Diseases published by Sanatatea Press Group on behalf of the Romanian Society of Diabetes Nutrition and Metabolic Diseases. This is an open access article under the terms of the Creative Commons Attribution License (CC-BY-NC-ND 3.0).

on elevating incretin hormones, DPP4i showed clinical significance to stimulate insulin release among patients with T2DM [4]. The use of DPP4i agents (sitagliptin, vildagliptin, linagliptin, and saxagliptin) in combination with other OADs or insulin is widely accepted because of their established tolerability and safety profiles [2].

In a study aimed to assess the effectiveness of DPP4i in clinical practice, the findings highlighted that sitagliptin use was associated with a significant reduction in HbA1c over the first six months in a pattern consistent with reported observations from clinical trials [5]. In addition, a systematic review looked at the extent of effectiveness of T2DM add-on therapies; the significant impact of DPP4i agents on attaining glycemic control was reported [6]. Furthermore, the use of DPP4i among patients with T2DM who had high cardiovascular and renal risk seems to be safe, and newer agents, e.g., linagliptin, may even be offered for renal patients with no need for dose adjustment [7]. Therefore, previous findings have underpinned a significant association between the use of DPP4i among patients with T2DM who were concurrently receiving cardiovascular medications such as beta-blockers and aspirin [8].

In most of the diabetes clinical guidelines, there are recommendations for the preferred use of specific OAD agents based on patients' characteristics and comorbidities [9]. Therefore, it is essential to investigate the quality use of OAD in the clinical setting by reporting the prescribing patterns and the associated achievement of favorable outcomes. In this research, we aimed to describe the prescription pattern of DPP4i among outpatients with T2DM at a university teaching hospital. Also, the assessment of the DPP4i use concerning the level of achievement of target HbA1c values was performed.

Material and Method

Study design and patients

A retrospective cross-sectional study was conducted. The data of patients receiving DPP4i in IIUMMC was collected from the hospital database through a prescribing period from March to September 2019 to ensure that the data of the latest HbA1c level of the patients could be obtained. We included data of adults with T2DM who received DPP4i prescriptions at least three months before the data collection and reported HbA1c after at least three months from the index date. Patients who received new in-ward DPP4i prescriptions and those who did not have reported HbA1c after at least three months from the index date were excluded. Initially, 1218 prescriptions of DPP4i during the period of data collection were screened. Following further exclusion of the in-ward prescriptions and patients not in line with the inclusion criteria, 191 prescriptions were identified. By considering the availability of the HbA1c results after at least three months, the final sample included 140 patients.

Outcome Measures

The assessment of the DPP4i use and the associated glycemic control achievement was determined according to the target levels classified by the Ministry of Health in the clinical practice guidelines (CPG) Management of Type 2 Diabetes Mellitus (5th Edition), as shown in Table 1.

Ethical Requirements

The study proposal has obtained ethical approval from the university institutional review board, IIUM Research Ethics Committee (IREC), before starting the data collection procedures. The study approval ID was IREC 2019–229. All ethical considerations were strictly abided by the research team throughout the research process.

Statistical analysis

Descriptive statistical analysis using frequencies and mean value for characteristics of the study population and HbA1c was used. In addition, inferential statistics using Chi-Square Test was employed to test for any significant

Table 1: Target HbA1clevels according to patients' characteristics and underlying conditions [2].

Individualized HbA1ctargets and patients' profile		
Tight (6.0–6.5%)	**6.6–7.0%**	**Less tight (7.1–8.0%)**
• Newly diagnosed • Younger age • Healthier (long life expectancy, no CVD complications) • Low risk of hypoglycemia	• All others	• Comorbidities (coronary disease, heart failure, renal failure, liver dysfunction) • Short life expectancy • Prone to hypoglycemia

association between the DPP4i types, prescribing pattern with the achievement of glycaemic control. The significant association was denoted at ($p<0.05$). All analyses were done using Statistical Package for the Social Sciences (SPSS) version 23.

Results

From the finally included sample of 140 patients receiving DPP4i in IIUMMC outpatient pharmacy, 61 patients were female, and 137 of them were Malay race. About 90% of participants received DPP4i as a combination therapy with other OAD. It was observed that 41.4% of males and 30.7% of female patients were given sitagliptin 50 mg. In comparison, the least prescribed DPP4i was vildagliptin 50 mg, with a percentage of 4.3% and 8.6% for females and males, respectively. There were no statistically significant differences between gender, race, and the type of prescribed DPP4i. Besides, most of the T2DM patients prescribed with DPP4i were in the age group of 58–67 years old (40%). There were no statistically significant differences between the age groups of the patients and the type of prescribed DPP4i. A large proportion of patients who were prescribed with sitagliptin 50 mg were overweight. normal. Meanwhile, obese patients were receiving more prescriptions of sitagliptin 100 mg. Figure 1 shows a graphical presentation of the prescribed DPP4i regimens according to the patients' body mass index (BMI). There was a statistically significant difference between the patient's BMI and the type of prescribed DPP4i (p-value = 0.024). The prescribing pattern of DPP4i concerning patients' characteristics is illustrated in Table 2.

Co-administered drugs with DPP4i

The findings showed that statin (85.7%) was the most frequently co-administered drug with DPP4i of the patients who were prescribed both statin and DPP4i. Next, antihypertensive therapy (72.9%) was the second most common co-administered drug with DPP4i. Besides, vitamins and supplements were found in 40% of the DPP4i prescriptions with a relatively higher percentage among vildagliptin users. Other medications, such as antiplatelet therapy and proton pump inhibitors, were also often co-administered with DPP4i in 35.8% and 20.7% of prescriptions, respectively. A similar percentage of patients (10%) were reported for anti-gout and anti-epileptic medications. Concerning the co-administered DM pharmacotherapy, metformin (64.1%) was the most common co-administered OAD with DPP4i, followed by gliclazide, and empagliflozin with reported percentages of 43.6% and 22.1%, respectively. For patients who were taking sitagliptin 50 mg and 100 mg, the most common co-administered OAD is metformin with a percentage of 47.9% and 12.9%, respectively. Meanwhile, gliclazide is the most common co-administered OAD for patients prescribed with vildagliptin 50 mg. Also, the most common insulin co-administered to the DPP4i receivers was Insulin Detemir (17.1%). In comparison, Ryzodeg "Insulin degludec 2.56 mg, insulin aspart 1.05 mg per ml" (2%) was the least common co-administered insulin among patients on DPP4i prescriptions.

Achievement of glycemic control

As an indicator of glycemic control in T2DM patients, the HbA1c levels of the included

https://doi.org/10.46389/rjd-2020-1057
© 2020 The Authors

Table 2: Prescribing pattern of DPP4i with patients' characteristics

Variable	Type of DPP-4 inhibitors			Total
	Sitagliptin 50 mg	Sitagliptin 100 mg	Vildagliptin 50 mg	
Prescription pattern				
Monotherapy	7 (5.0%)	0	7 (5.0%)	14 (10%)
Combination therapy	94 (67.1%)	21 (15.0%)	11 (7.9%)	126 (90%)
Gender				
Female	43 (30.7%)	12 (8.6%)	6 (4.3%)	61 (43.6%)
Male	58 (41.4%)	9 (6.4%)	12 (8.6%)	79 (56.4%)
Race				
Malay	99 (70.7%)	21 (15.0%)	17 (12.1%)	137 (97%)
Chinese	0	0	1 (0.7%)	1 (0.7%)
Indian	2 (1.4%)	0	0	2 (1.4%)
Age				
28–37 years	2 (1.4%)	0	0	2 (1.4%)
38–47 years	9 (6.4%)	1 (0.7%)	2 (1.4%)	12 (8.6%)
48–57 years	27 (19.3%)	7 (5.0%)	5 (3.6%)	39 (27.9%)
58–67 years	41 (29.3%)	10 (7.1%)	5 (3.6%)	56 (40%)
68–77 years	20 (14.3%)	3 (2.1%)	3 (2.1%)	26 (18.6%)
78–87 years	2 (1.4%)	0	3 (2.1%)	5 (3.6%)
Body Mass Index (BMI)				
<18 (underweight)	0	0	0	0
18.5–24.9 (normal)	23 (16.4%)	3 (2.1%)	5 (3.6%)	31 (22.1%)
25–29.9 (overweight)	41 (29.3%)	5 (3.6%)	5 (3.6%)	51 (36.4%)
>30 (obese)	31 (22.1%)	11 (7.9%)	3 (3.6%)	45 (32.1%)

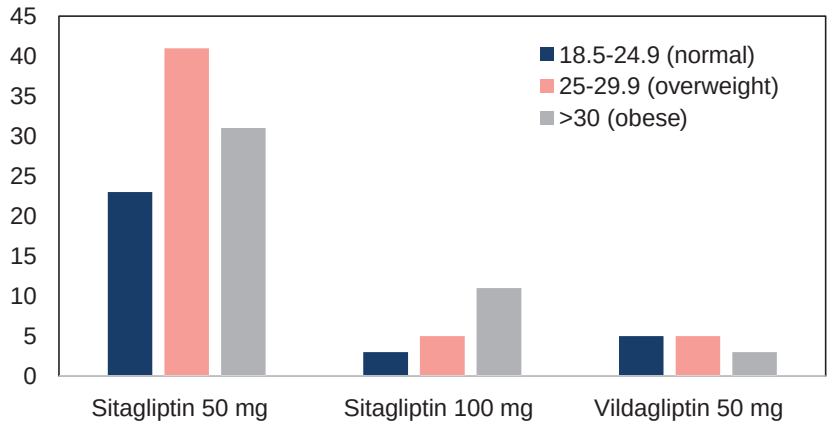


Figure 1: Prescribing pattern of DPP-4 inhibitors according to the BMI of the patient

patients were recorded. The mean values of HbA1c before the initiation of DPP-4 inhibitor therapy, after three months and six months, were 9.0 ± 2.2, 8.0 ± 2.0, and 8.2 ± 1.9, respectively. The result shows that 35% (N=49) of the patients taking DPP-4 inhibitors achieved within target values of HbA1c. Table 3 shows the overall association of DPP4i regimens with their reported HbA1c measures.

Among those 101 patients who are on current sitagliptin 50 mg prescription, only 36.6% of them manage to reach the HbA1c target, whereby the patients who were given combination therapy with other OAD contributed the highest percentage (22.8%). Also, about 45% of patients who were taking vildagliptin 50 mg had achieved the HbA1c target; of them, 33.3% were on monotherapy. Table 4 demonstrates the details of the

© 2020 The Authors

Table 3: Target achievement of the HbA1c levels.

Type of DPP-4 inhibitors	HbA1clevel within the target		HbA1clevel out of the target	
	Frequency	Percentage (%)	Frequency	Percentage (%)
Sitagliptin 50 mg	37	26.4	64	45.8
Sitagliptin 100 mg	4	2.9	17	12.1
Vildagliptin 50 mg	8	5.7	10	7.1
Total	**49**	**35.0**	**91**	**65.0**

Table 4: Target of HbA1c level achieved with the use of DPP-4 inhibitors.

Prescribing pattern	Percentage of patients that reach the HbA1c target (%)		
	Sitagliptin 50 mg (n= 37 out of 101)	Sitagliptin 100 mg (n= 4 out of 21)	Vildagliptin 50 mg (n= 8 out of 18)
Monotherapy	5.9	0	33.3
Combination therapy with insulin only	2.0	0	11.1
Combination therapy with other OAD only	22.8	19.0	0
Combination with insulin and other OAD	5.9	0	0
Percentage of patients reach HbA1c target (%)	36.6	**19.0**	**44.4**

Table 5: A summary of variables with a significant association with the type of DPP4i

Variables	P-value (p<0.05)
Prescription pattern	0.000
Body Mass Index (BMI)	0.024
Co-administered drug (vitamin and supplement)	0.008
Co-administered OADs (metformin)	0.000

prescribed DPP4i patterns within the cases that have achieved HbA1c values. Finally, the variables listed in Table 5 showed statistically significant association with the type of the prescribed DPP4i

Discussion

Type of DPP-4 Inhibitors Therapy Commonly Prescribed in the Study Setting

Sitagliptin and vildagliptin were the only two types of prescribed DPP4i in the study setting. Overall results showed more prescriptions of DPP4i combination therapy compared to monotherapy. It might be explained by the superior HbA1c reduction of sitagliptin/metformin combination compared to monotherapy of each drug [10]. Also, it was reported that the use of sitagliptin 100 mg as add-on therapy to 1000 mg metformin reduced HbA1c by 0.9% over 24 weeks among Chinese patients with T2DM following inadequate glycemic control with metformin alone [11]. Moreover, it is well established that metformin is the mainstay in the monotherapy treatment initiation, and other OAD, e.g., DPP4i, will be considered in case of inadequate control [2]. Therefore, the patterns observed in this study seem to be in line with the clinical guidelines.

Vildagliptin was the least prescribed DPP4i among our study participants despite the evidence that its associated HbA1c reduction was comparable to that of sitagliptin [12]. Also, the percentage of patients on vildagliptin monotherapy was relatively higher than that of siltagliptin monotherapy. In contrast, a previous study reported an overall low utilization of vildagliptin monotherapy [8]. The prescribing of DPP4i

https://doi.org/10.46389/rjd-2020-1057
© 2020 The Authors

monotherapy could be noticed among patients with renal problems and those with severe heart failure in whom metformin therapy will not be an appropriate therapeutic choice [13].

Common Co-Administered Drugs with DPP-4 Inhibitors

Vildagliptin was frequently prescribed for the patient receiving calcitriol or ferrous fumarate supplement. The dosage of vildagliptin should be halved from 100 mg twice daily to 50 mg once daily in a patient with moderate to severe renal impairment [14]. Although our work did not trace for the concurrent comorbidities extensively, it is noticeable that these supplements for vitamin D deficiency and iron-deficiency anemia can be seen in a patient with chronic kidney disease (CKD). Moreover, vildagliptin is more preferred in a patient with CKD compared to sitagliptin due to its favorable pharmacokinetic properties [14, 15]. Only 23% of orally ingested vildagliptin excreted unchanged in the urine, whereas most of the ingested sitagliptin exclusively excreted unchanged in the urine [14].

Next, DPP4i was frequently prescribed in combination therapy with gliclazide in line with the recommendations of CPG on the management of T2DM [2]. Gliclazide is a preferred sulfonylurea as a second-line OAD because of its relatively safer CV profile for and less incidence of hypoglycemia compared to the other sulfonylureas [16]. In contrast, glibenclamide has shown an undesirable effect on type 2 diabetes mellitus patients with coronary artery disease [17]. From the medications screening, it was clear that many patients had a comorbid cardiovascular disease, which could explain the frequent use of DPP4i in combination therapy with gliclazide.

Type of the Prescribed DPP4i among Patients with Different BMI

According to the statistics released by the National Diabetes Registry Report in 2012, 83.4% of the diabetic patients were also overweight or obese [2]. Thus, a prescriber needs to consider the impact of OAD on weight gain. According to the national clinical practice guidelines for the management of T2DM, metformin is the first-line agent for patients with normal, overweight, and obese BMI. DPP-4 inhibitor is considered a second-line choice for normal and overweight patients and the fourth line in obese [2]. The findings showed frequent use of sitagliptin, which is preferred in overweight patients compared to thiazolidinediones and sulphonylureas due to its weight neutral properties [5]. However, for obese patients, other antidiabetic agents such as SGLT2i and GLP-1 receptor antagonists that could promote better glycemic control in obese patients compared to sitagliptin [18]. It is suggested that the choice of the add-on OAD therapies should consider the individual patients' conditions carefully to give the optimal glycemic control and avoid unwanted health outcomes.

The Level of HbA1c Target Achievement with DPP-4 Inhibitors Therapy

Overall, the use of both sitagliptin and vildagliptin can achieve glycemic control. Based on the previous study, 66% of patients who received sitagliptin 100 mg as combination therapy with metformin 2000 mg achieved HbA1c target less than 7% [19]. In comparison with our findings, the prescribed doses were relatively lower. Nevertheless, a still considerable number of patients on sitagliptin combination therapy could achieve glycemic control.

Among the relatively low number of cases on vildagliptin therapy, the majority has achieved the target HbA1c. This was consistent with prior evidence regarding the extent of attaining target HbA1c after four months on vildagliptin monotherapy [20]. With greater focus, it has been reported in our findings that a more substantial proportion of patients failed to achieve target HbA1c. Considering the lack of investigation of all the barriers related to the achievement of target HbA1c, we could only suggest that better glycemic control could be achieved through

© 2020 The Authors

addressing patients' non-compliance to medications, insufficient treatment, and non-adherence to therapeutic lifestyle changes.

Conclusions

The DPP4i were more prescribed as combination therapy, mainly with metformin, compared to monotherapy except for vildagliptin. Gliclazide was the most common co-administered OAD with vildagliptin. Overall, only one-third of patients prescribed with DPP4i were able to achieve target HbA1c values. Barriers to achieving optimal glycemic control for patients on OAD, particularly DPP4i, need further investigations.

Limitations

There were several limitations upon the completion of this research. One limitation was time constraints because of the short period specified for executing all data collection procedures. Also, there was a difficulty in accessing the data of patients before they started to receive diabetes care in the hospital, which may affect the accuracy of the data related to the exact date of DPP4i initiation.

Conflict of Interest

The authors declare no conflict of interest.

References

1. Tee ES, Yap RWK. Type 2 diabetes mellitus in Malaysia: Current trends and risk factors. *Eur J Clin Nutr*. 71: 844–849, 2017.
2. Health M of. *Clinical Practice Guidline: Management of Type 2 Diabetes Mellitus*. 5th ed. Putrajaya: Ministry of Health Malaysia, 2015.
3. Sherwani SI, Khan HA, Ekhzaimy A, et al. Significance of HbA1c test in diagnosis and prognosis of diabetic patients. *Biomark Insights*. 11: 95–104, 2016.
4. Gallwitz B. Clinical use of DPP-4 inhibitors. *Front Endocrinol (Lausanne)*. 10: 1–10, 2019.
5. Dallumal RM, Chua SS, Wu DBC, et al. Sitagliptin: Is it effective in routine clinical practice? *Int J Endocrinol*. 2015: 1–9, 2015.
6. Wong MCS, Wang HHX, Kwan MWM, Zhang DDX, Liu KQL, Chan SWM, et al. (2014) Comparative Effectiveness of Dipeptidyl Peptidase-4 (DPP-4) Inhibitors and Human Glucagon-Like Peptide-1 (GLP-1) Analogue as Add-On Therapies to Sulphonylurea among Diabetes Patients in the Asia-Pacific Region: A Systematic Review. PLoS ONE 9(3): e90963. https://doi.org/10.1371/journal.pone.0090963
7. Hanssen NMJ, Jandeleit-Dahm KAM. Dipeptidyl peptidase-4 inhibitors and cardiovascular and renal disease in type 2 diabetes: What have we learned from the CARMELINA trial? *Diabetes Vasc Dis Res*. 16: 303–309, 2019.
8. Hasniza Zaman Huri, NorFarahen Selamat, Shireene Ratna Vethakkan, "Factors Associated with Utilization of Dipeptidyl-4 Inhibitors in Patients with Type 2 Diabetes Mellitus: A Cross-Sectional Retrospective Study", *International Journal of Endocrinology*, vol. 2014, Article ID 367564, 7 pages, 2014. https://doi.org/10.1155/2014/367564
9. Reusch JEB, Manson JE. Management of Type 2 Diabetes in 2017. *Jama*. 317: 1015, 2017.
10. Hayes J, Anderson R, Stephens JW. Sitagliptin/metformin fixed-dose combination in type 2 diabetes mellitus: An evidence-based review of its place in therapy. *Drug Des Devel Ther*. 10: 2263–2270, 2016.
11. Yang W, Guan Y, Shentu Y, et al. The addition of sitagliptin to ongoing metformin therapy significantly improves glycemic control in Chinese patients with type 2 diabetes. *J Diabetes*. 4: 227–237, 2012.
12. Marfella R, Barbieri M, Grella R, et al. Effects of vildagliptin twice daily vs. sitagliptin once daily on 24-hour acute glucose fluctuations. *J Diabetes Complications*. 24: 79–83, 2010.
13. Davoren P. Safe prescribing of metformin in diabetes. *Aust Prescr*. 37: 2–5, 2014.
14. He YL, Kulmatycki K, Zhang Y, et al. Pharmacokinetics of vildagliptin in patients with varying degrees of renal impairment. *Int J Clin Pharmacol Ther*. 51: 693–703, 2013.
15. Ministry of Health (New Zealand). *Medsafe: DPP4 inhibitors*. 2018.
16. Fadini GP, Bottigliengo D, D'Angelo F, et al. Comparative Effectiveness of DPP-4 Inhibitors Versus Sulfonylurea for the Treatment of Type 2 Diabetes in Routine Clinical Practice: A Retrospective Multicenter Real-World Study. *Diabetes Ther*.9: 1477–1490, 2018.
17. Azimova K, San Juan Z, Mukherjee D. Cardiovascular safety profile of currently available diabetic drugs. *Ochsner J*. 14: 616–632, 2014.
18. Kodera R, Shikata K, Nakamura A, et al. The glucose-lowering efficacy of sitagliptin in obese japanese patients with type 2 diabetes. *Intern Med*. 56: 605–613, 2017.
19. Barry J, Goldstein M, Johnson J, MD B 3, Mark N, Feinglos M, et al. Effect of Initial Combination Therapy With Sitagliptin, a Dipeptidyl Peptidase-4 Inhibitor, and Metformin on Glycemic Control in Patients With Type 2 Diabetes. *Diabetes Care*; 30. Epub ahead of print 2007. DOI: 10.2337/dc07-0627. Clinical.
20. Yavropoulou MP, Pikilidou M, Kotsa K, et al. Efficacy and tolerability of vildagliptin as first line treatment in patients with diabetes type 2 in an outpatient setting. *J Diabetes Metab Disord*. 14: 1–7, 2015.

 https://doi.org/10.46389/rjd-2020-1057 © 2020 The Authors

https://doi.org/10.46389/rjd-2020-1058
www.rjdnmd.org

Rom J Diabetes Nutr Metab Dis
2020; volume 27, issue 4, pages 403-409

Review

Inherited or acquired in hypertension and chronic kidney disease in diabetes mellitus patients

Stoian Marilena MD[1,2]*, Dumitrache Ana Maria[2], Cîrciu Fivi[2], Stoica Victor MD[1,2], Radulian Gabriela MD[1,3]

[1] "Carol Davila" University of Medicine and Pharmacy, Bucharest, Romania
[2] Internal Medicine Department, "Dr. Ion Cantacuzino" Clinical Hospital, Bucharest, Romania
[3] N.C. Paulescu National Institute for Diabetes, Nutrition and Metabolic Diseases, Bucharest, Romania

*Correspondence to: Stoian Marilena MD, Matei Basarab Street, L109, sc 2, ap 27, District 2, P.O. 030675, Bucharest, Romania, E-mail: marilenastoian@yahoo.com, Phone: +400733937310

Received: 6 September 2020 / Accepted: 26 November 2020

Abstract

Diabetic kidney disease (DKD) is a common and serious microvascular complication of diabetes mellitus (DM), which is characterized by an elevated urinary albumin excretion rate, elevated blood pressure, and declined renal function. Approximately 30–40% of DM patients will develop DKD, which is the leading cause of end-stage renal disease (ESRD) and renal failure. Genetic factors appear critical in DKD pathogenesis based upon the evidence including aggregation in families, variable incidence rates of DKD between different races, and the highly heritable nature of diabetic renal clinic and histologic changes. Each 10-mmHg increase in mean systolic blood pressure (BP) was associated with a 15% increase in the hazard ratio for development of both micro- and macroalbuminuria and impaired kidney function defined as eGFR<60 ml/min per 1.73 m^2 or doubling of the blood creatinine level. Broadly, a baseline systolic BP >140 mmHg in patients with DM2 has been associated with higher risk of ESRD and death. The ACE genes may predict diabetic nephropathy in some groups, the rate of progression and the antiproteinuric response to ACE inhibitors.

Keywords: *ACE* genes, diabetic kidney disease, diabetes mellitus, hypertension, genetic susceptibility.

Diabetic kidney diseases definition

Diabetic kidney disease (DKD) is one of the major chronic complications of diabetes, associated with significant morbidity and mortality, with rates of hospitalization three times higher in patients with DKD than in patients without renal complication [1]. In diabetic kidney disease, the progressive decline of glomerular filtration rate (GFR) towards end stage renal disease (ESRD) which is associated with increased mortality, is mainly due to cardiovascular causes [2]. It is postulated that reduced renal function is by itself an indicator of high cardiovascular mortality risk. [3]. DKD is a clinical syndrome characterized by: increased urinary albumin excretion rate (UAER) that is confirmed on at least two occasions three months apart; progressive decline in the glomerular filtration rate (GFR); elevated arterial blood pressure without other renal disease or heart failure [4, 5]. UAER is defined as an excretion rate below 20 µg/min or 30 mg/24 hours [5]. An excretion rate between 20-200 µg/min and 30–300 mg/24 hours defines microalbuminuria or more recently named "*moderately increased albuminuria*" [6]. An excretion rate >200 microgame (300 mg/24 hours) is named macroalbuminuria or conformed recently to recommendations "*severe increased albuminuria*" [6]. An alternative method of detecting microalbuminuria is measurement

© 2020 The Authors. Romanian Journal of Diabetes, Nutrition and Metabolic Diseases published by Sanatatea Press Group on behalf of the Romanian Society of Diabetes Nutrition and Metabolic Diseases. This is an open access article under the terms of the Creative Commons Attribution License (CC-BY-NC-ND 3.0).

of the albumin/creatinine ratio in a spot urine specimen: a ratio between 30–300 mg albumin/g creatinine is well correlated with 24-hours collections, and is now the preferred screening test for diabetic kidney disease [7]. Microalbuminuria as a marker of glomerular damage predicts the development of overt nephropathy without specific interventions in approximately 80% of insulin-dependent diabetes mellitus, and 20–40% of patients with non-insulin-dependent diabetes mellitus. Moderately increased albuminuria is also a marker of increased cardiovascular morbidity and mortality in patients with either type 1 diabetes (T1DM) or type 2 diabetes (T2DM). Some evidence also indicates that moderately increased albuminuria may predict cardiovascular events and perhaps early renal damage in patients with essential hypertension [8]. GFR is calculated using MDRD and CKD-EPI equations [6]. Characteristic structural and functional changes in diabetic kidney disease include hyperfiltration, renal and glomerular hypertrophy, mesangial cell hypertrophy and matrix accumulation, glomerular basal membrane thickening, and functional alteration in glomerular filtration barriers. Factors responsible for these typical changes are hyperglycemia, advanced glycosylation end-products (AGEs), growth factors, cytokines, and glomerular hypertension [9]. DKD pathogenesis involves both genetic and environmental factors represented by older age, male sex, smoking status, and ethnic background (African-American, Native American, and Mexican-American people have a much higher risk -three- to six-fold increase – of developing end-stage renal disease in the setting of diabetes compared with white Caucasian subjects) [10]. The genetic risk of DKD is influenced by the combined effects of variation at an undetermined number of genomic sites, some with a predisposing and some with a protective effect [11, 12]. The screening for DKD should be performed at diagnosis in T2DM and five years after initial diagnosis in T1DM with a urinary albumin/creatinine ratio and serum creatinine. The patients with diabetes mellitus whose GRF decline rate is exceeding 10 ml/min/year or is lower than 30 ml/min should be referred immediately for nephrological exam.

Hypertension in diabetes mellitus

Hypertension in diabetes mellitus may be due to one of the following reasons: the metabolic syndrome (hypertension, obesity, atherosclerosis, and dyslipidemia); secondary to complications of diabetes mellitus; due to endocrine disorders and drugs, and coincidental (essential arterial hypertension and isolated systolic hypertension). The renin-angiotensin-aldosterone system (RAS), natriuretic peptides system (NPS), Endothelin I, Bradykinins and NO are important regulators of fluid and electrolytes homeostasis. The balance between different endogenous vasoconstrictors (angiotensin II, norepinephrine, endothelin-1) and vasodilators (natriuretic peptides, bradykinin, adrenomedullin, and NO) is alterated in diabetes patients and predisposes to hypertension and microvascular diseases, including diabetic kidney disease. The existence of intricating mechanisms between hypertension and hyperglycemia is sustained by two observations:

- the diabetic patients, especially after onset of chronic complications, have a higher prevalence of hypertension than non-diabetic controls [13];
- the risk of diabetic kidney disease increases in subjects with a family history for hypertension or cardiovascular disease [14, 15].

It is estimated that the risk for loss of renal function is several times higher in hypertensive diabetic patients than in hypertensive nondiabetic patients [15]. In patients with newly diagnosed DM2, treating to a target BP of <150/85 mmHg over a median of 15 years resulted in a significant 37% risk reduction of microvascular complications compared with that in patients treated to a target of <180/105 mmHg. Each 10-mmHg increase in mean systolic BP was associated with a 15% increase in the hazard ratio for development of both micro- and macroalbuminuria and impaired kidney function defined as eGFR <60 ml/min per 1.73 m^2 or doubling of the blood creatinine level [16]. Broadly, a baseline systolic BP >140 mmHg in patients with DM2 has been associated with higher risk of ESRD and

 https://doi.org/10.46389/rjd-2020-1058 © 2020 The Authors

death [17, 18]. The natural history of hypertension differs markedly between T1DM and T2DM. In insulin-dependent diabetes mellitus patients the blood pressure is usually normal at presentation and remains normal for the first 5-10 years, but increases with the appearance of diabetic kidney disease. To the non-insulin-dependent diabetes mellitus patients, elevated blood pressure is usually present at diagnosis or diabetes, or may develop thereafter [19]. Systemic hypertension is an early phenomenon in diabetic kidney disease. Furthermore, nocturnal blood pressure elevation ("non-dippers") occurs more frequently in insulin-dependent diabetes mellitus and non-insulin-dependent diabetes mellitus patients with diabetic kidney disease. Also, exaggerated blood pressure response to exercise has been reported in long-standing insulin-dependent diabetes mellitus patients with microangiopathy. Finally, the increase in glomerular pressure consequent to nephron adaptation may be accentuated with concomitant diabetes [9]. The risk for progression for DKD is reduced with reduction of blood pressure to less than 130-140/80-85 mmHg and optimization of HbA1c. In the last 25 years ,the hypothesis that renin -angiotensin system (RAS) inhibitors, either inhibitors of the angiotensin-converting enzyme (ACE-I) or angiotensin receptors blockers (ARB) prevent the development or slow the progression of diabetic kidney disease was intensely studied [20-23]. Clinical and experimental data reveal that blocking RAS with ACE-Is or ARBs reduces transition from microalbuminuria to proteinuria. What do we do, now? We think that Is rationale To use an anti-hypertensive with renal protective agents with dual blockade; in fact, neither ACE-Is or ARBs can completely suppress the RAS, because of the "escape" phenomenon: alterate nonACE-dependent pathways (chymase) for angiotensinogen activation to angiotensin II. These mechanisms are probably responsible for the return of angiotensin II levels back to baseline after six to nine months of ACE-I treatment [24]. A multitude of association studies of blood pressure candidate genes was performed; we resume five physiological genes classes: renin-angiotensin-aldosterone system(RAS), sodium volume, sympathetic nervous system, vascular and metabolic systems [25, 26].

Genetic susceptibility to diebetic kidney disease

DKD is a complex, multifactorial syndrome, which develops when environmental risk factors operate on a pool of genetic variants that confers individual susceptibility to disease. A high number of genes are considered candidates for DKD, an explanation for this high number resides in the intrinsic complexity of the pathophysiology of diabetic renal injury (Table 1 and 2). The number of DKD candidate genes will increase in the following years while more data regarding the cellular and molecular pathophysiology of the initial phases of DKD will become available. According to the presumed molecular mechanism of their encoded protein, these genes are grouped into:

- **Blood pressure regulation genes:** Angiotensin Converting Enzyme-ACE, Angiotensinogen-AGT, Angiotensin Receptors-AGTR, Aldosterone Syntetase-CYP11B2, Endothelin and Endothelin Receptors, Plasma Kallikrein Bradykinin Receptors, Nitric Oxide Synthetases-NOS2A, NOS3;
- **Growth factors and angiogenesis factors:** Vascular Endothelial Growth Factor-VEGF, Erythropoetin, TGFβ;
- **Metabolism genes:** Aldolase Reductase-AKR1B1, AGE Receptor-AGER, Apolipoproteins, Methylenetetrahydrofolate reductase-MTHFR;
- **Cytokines and inflammation genes:** IL-1. IL6, IL18, ICAM1, MCP1, TNFα;
- **Oxidative stress:** Nitric Oxide Synthetases, Glutathione Peroxidase-GPX, Catalase-CAT;
- Genes with other function: HSPG2-Heparin sulfate Proteoglycan which Is involved in glomerular structure, GREM1 which is involved in cell growth, UNC13B thought to be involved in apoptosis).

In the last two decades, the attention has focused on genetic susceptibility to renal injury from elevated blood pressure. According to Churchill et al [27], an experimental animal model of renal injury caused by hypertension suggests that nephropathy susceptible genes exist, but genes have not yet been identified. In humans, the familial clustering of hypertensive renal

© 2020 The Authors

Table 1: Candidate genes for DKD: adapted after [31]

Gene Class	Gene	Location	Loci	Population	Phenotype
Cytokines and growth factors	Adiponectin	3q	ADIPOQ	Danish, Finnish, French	Type 1 DN
	IGF-1	12q23.2	IGF1	White	Type 1 DN
	IGF-binding protein 1	7p14	IGFBP1	White	Type 2 DN
	TGF-β receptor II	3p24.1	TGFβR2	White	Type 1 DN
	TGF-β receptor III	1p22.1	TGFβR3	White	Type 1 DN
Extracellular matrix components	Collagen type IV, α I	7q32.1	COL4AI	White	Type 1 DN
	Laminin, α 4	6q21	LAMA4	White	Type 1 DN
	Laminin, γ 1	1q25.3	LAMCI	White	Type 1 DN
Matrix metalloproteinases and dipeptidases	Tissue inhibitor of metalloproteinase 3	22q12.3	TIMP3	White	Type 1 DN
	Matrix metalloproteinase 9	20q13.12	MMP9	White	Type 1 DN
	Carnosinase	18q22.3	CNDP1	White	Type 2 DN
Transcription factors	HNF1B1/transcription factor 2, hepatic (MODY5)	17q12	HNF1B1/ TCF2	White	Type 1 DN
	Neuropilin 1	10p11.22	NRPI	White	Type 1 DN
	Protein kinase C β 1	16p12.1	PRKCBI	White	Type 1 DN
	SMAD, mothers against DPP homolog 3	15q22.33	SMAD3	White	Type 1 DN
	Upstream transcription factor 1	1q23.3	USFI	White	Type 1 DN
Renal function and renin angiotensin system components	Angiotensin II receptor, type 1	3q24	AGTR1	White	Type 1 DN
	Aquaporin 1	7p14.3	AQP1	White	Type 1 DN
	B-cell leukemia/lymphoma 2 (bcl-2) proto-oncogene	18q21.33	BCL2	White	Type 1 DN
	Catalase	11p13	CAT	White	Type 1 DN
	Glutathione peroxidase 1	3p21.3	GPXI	White	Type 1 DN
	Lipoprotein lipase	8p21.3	LPL	White	Type 1 DN
	Cytochrome b, α polypeptide	16q24.3	p22phox	White	Type 1 DN
	Angiotensin-converting enzyme	17q23	ACE	White	Type 1 DN, Type 2 DN
Inflammatory factors	Engulfment and cell motility factor	7p14	ELMO1	Japanese, Black	Type 2 DN
Endothelial function and oxidative stress	Nitric oxide synthase 3	7q36.1	NOS3	Japanese, White	DN, Type 1 DN
	Superoxide dismutase 2	6q25	SOD2	Caucasian, Korean, Japanese	Type 1 DN, Type 2 DN
Lipid metabolism	Apolipoprotein E	19q	ApoE	White	Type 1 DN, Type 2 DN

https://doi.org/10.46389/rjd-2020-1058
© 2020 The Authors

Table 2: Genome-wide linkage scans for diabetic kidney disease: adapted after [31]

Chromosome	Region	Maximum LOD	Population	Study	Phenotype	Characteristics
3q	13	4.55	Black	Sibling pairs	Type 2 DN	Age at ESRD onset
	21.3	2.67	Finnish	Discordant sibling pairs	Type 1 DN	
	25.1	3.1	White	Discordant sibling pairs	Type 1 DN	
7q	12.3	1.84	West African	Sibling pairs	Type 2 DN	CC
	21.1	(6.0×10^{-4})	White	90% sibling pairs	Predominantly type 2 DN	ACR
	21.3	(6.0×10^{-5})	Black	90% sibling pairs	Predominantly type 2 DN	Nephropathy
	33	2.04 to 2.73	Pima Indian	Sibling pairs	Type 2 DN	Nephropathy and retinopathy
	36.2	3.1	94% white	Families	Type 2 DN	ACR
	(99 cM)	(1.1×10^{-4})	White	90% sibling pairs	Predominantly type 2 DN	Nephropathy
7p	21.3	4	94% white	Sibling pairs	Type 2 DN	CC-GFR
	32.1	3.59	Black	Sibling pairs	Type 2 DN	Age at diabetes onset
	(12 cM)	(1.6×10^{-4})	American Indian	90% sibling pairs	Predominantly type 2 DN	ACR
	(78 cM)	(1.0×10^{-3})	Mexican American	90% sibling pairs	Predominantly type 2 DN	GFR
10q	23.31	3.1	94% white	Sibling pairs	Type 2 DN	Diabetic/ nondiabetic; CC-GFR
	26	2.47	Black	Sibling pairs	Type 2 DN	Age at ESRD onset
18q	22.1	3.72	Black	Sibling pairs	Type 2 DN	Age at diabetes onset
	22.1	(3.15×10^{-2})	White	Discordant sibling pairs	Predominantly type 2 DN	Nephropathy
	22.3–23	6.1	Turkish	Families	Type 2 DN	Nephropathy

disease and the identification of polymorphism in the renin-angiotensin-aldosteron system gene components support the idea of genetic susceptibility to hypertensive renal injury in diabetic nephropathy [28]. Krolewski et al [29] identified a region on the long arm of chromosome 3 in the vicinity of the angiotensin II type-1 receptor gene that harbors a locus with major effects. In addition, they have demonstrated minor effects of the insertion allele in the ACE gene and the T-allele at position 235 in the angiotensinogen gene on the development on diabetic nephropathy; this

© 2020 The Authors

finding must be confirmed in other family-based studies.

Is susceptibility to diabetic nephropathy the same as susceptibility to essential hypertension? Krolewski et al [29] identified some overlap. Essential hypertension has a significant genetic component with minor gene affects and these authors postulated that the expression and penetrance of one of these minor genes for essential hypertension is changed in the presence of hyperglycemia in such a way that carriers of that disease allele, which must be a common one, would develop diabetic nephropathy together with their hypertension. Overactivity of the natrium/kalium exchanger in the pathogenesis of diabetic nephropathy remains uncertain. Demanine et al [30] presented the results of an analysis of polymorphism in two areas of the aldose reductase gene in normal healthy controls and in insulin-dependent diabetes mellitus patients with nephropathy; this finding is incert by others authors [29].

The kidney also plays a critical role in the development of systemic hypertension. The major alterations are sodium retention and increased peripheral vascular resistance [28]. The molecular aspect of this phenomenon in patients with diabetic nephropathy is not completely understood.

Conclusion

Hypertension and microalbuminuria play a critical role in initiation and progression of diabetic kidney disease. The ACE genes may predict diabetic nephropathy in some groups. Insulin resistance contributes to diabetic nephropathy but mostly indirectly. ACE genes may predict the rate of progression and the antiproteinuric response to ACE inhibitors. Diabetic kidney disease does not develop in the absence of hyperglycemia but other factors exist that interact with poor glycemic control to produce nephropathy and hypertension. Genetic susceptibility is one of the most important factors. The detection of genetically predisposed subjects will improve the results of the preventive strategies.

Conflict of Interest

The authors declare no conflict of interest.

References

1. van Gelder VA, Scherpbier-de Haan ND, de Graw WJ, O·Callaghan CA, Wetzels JF, Lasserson DS. Impact on cardiovascular risk follow-up from a shift to the CKD-EPI formula for eGFR reporting: a cross-selectional population-based primary care study. *BMJ Open*. 3(9):e003631, 2013.
2. Wong MG, Pollock CA, Cooper BA, Branley P, Collins JF, Criag JC, Kesselhut J, et al. Association between GFR estimated by multiple methods at dialysis commencement and patient survival. *Clin J Am Soc Nephrol*. 31, 2013.
3. Pagano E, Bo S, Petrico M, Rosato R,Merletti F, Gregori D. Factors affecting hospitalization costs in type 2 diabetic patients. *J Diabetes Complications*. 23:1–6, 2009.
4. Tang SC, Chan GC, Lai KN. Recent advances in managing and understanding diabetic nephropathy. *F1000Res*. 5, 2016.
5. Mogensen CE. Definition of diabeticrenal disease in insulin dependent diabetes mellitus,based on renal function tests. In: *The Kidney and Hypertension in Diabetes Mellitus*. Mogensen Ce (ed). Kluwer Academic Publishers, Boston, USA. 13–29, 2003.
6. KDIGO.KDIGO 2012 clinical practice guideline for the evaluation and management of chronic kidney disease. *Kidney Int Supplements*. 3:15–18, 2013.
7. Eknoyan G, Hostetter T, Bakris GL, Hebert L, Levey AS, Parving HH, Steffes Mw,Toto R.Proteinuria and other markers of chronic kidney disase:a position statement of yhe National Kidney Foundation (NKF) and the National Institute of Diabetes and Digestive and Kidney Diseases(NIDDK). *Am J Kidney Dis*. 42:617–622, 2003.
8. American Diabetes Association. *Clinical Practice Recommendations*. 23(Suppl), 2000.
9. Parving H, Osterby R, Anderson P et al. Diabetic nephropathy. In: Brenner B, ed. *The Kidney*. WB Saunders Co, Philadelphia.1874–1875, 1996.
10. Smith SR, Svetkey LP, DennisVW.Racial differences in the incidence and progression of renal diseases. *Kidney Int*. 40:815–822, 1991.
11. Cimponeriu D, Craciun AM, Apostol P, Radu I, Guja C, Cheta D. The genetic background of diabetes chronic complications. In: *Genetics of diabetes. The Thrth Unveiled*. Cheta D (ed. ED Acad Rom & S, Karger AG, Bucharest/Basel. 193–334, 2001.
12. Freedman BI, Bostrom M, Daeihagh P, Bowden DW. *Genetic factors in diabetic nephropathy*. 2(6):1306–1316, 2007.
13. Bretzel RG. Effects of antihypertensive drugs on renal function in patients with diabetic nephropathy. *Am. J Hypertens*. 10:208S–217S, 1997.
14. Roglic G, Cohoun HM, Stevens LK, Lemkes HH, Manes C, Fuller JH. Parenteral history of hypertension and parental history of diabetes and microvascular complications in insulin-dependent diabetes mellitus:the EURODIAB IDDM Complications Study. *Diabet Med*. 15:418–426, 1998.
15. De Cosmo S, Bacci S, Piras GP, Cignarelli M, Placentino G, Margaglione M, Colaizzzo D, et al. High prevalence of risk

 https://doi.org/10.46389/rjd-2020-1058 © 2020 The Authors

factors for cardiovascular disease in parents of IDDM patients with albuminuria. *Diabetologia*. 40:1191–1196, 1997.
16. Retnakaran R, Cull CA, Thorne KI, Adler AI, Holman RR; UKPDS Study Group: Risk factors for renal dysfunction in type 2 diabetes: U.K. Prospective Diabetes Study 74. *Diabetes*. 55: 1832–1839, 2006.
17. Bakris GL, Weir MR, Shanifar S, Zhang Z, Douglas J, van Dijk DJ, Brenner BM; RENAAL Study Group: Effects of blood pressure level on progression of diabetic nephropathy: Results from the RENAAL study. *Arch Intern Med*. 163: 1555–1565, 2003.
18. Pohl MA, Blumenthal S, Cordonnier DJ, De Alvaro F, Deferrari G, Eisner G, Esmatjes E, et al. Independent and additive impact of blood pressure control and angiotensin II receptor blockade on renal outcomes in the irbesartan diabetic nephropathy trial: Clinical implications and limitations. *J Am Soc Nephrol*. 16: 3027–3037, 2005.
19. Ayaeha A, Motala MD. Management of hypertension in diabetes mellitus. *JIMSA*.3:1–9, 1997.
20. Lewis Ej, Hunsicher LG,Clarke WR, et al.Renoprotective effect of angiotensin receptor antagonist irbesartan in patients with nephropathy due to type 2 diabetes. *N Engl J Med*. 345:851–860, 2001.
21. Brenner BM, Cooper ME, de Zeeuw D, et al. Effects of losartan on renal and cardiovascular outcomes in patienys with type 2 diabetes and nephropathy. *N Engl J Med*. 345:861–869, 2001.
22. Mauer M, Zinman B, Gardiner R, et al. Renal and retinal effects of enalapril and losartan in type 1 diabetes. *N Engl J Med*. 361:40–51, 2009.
23. Fried LF, Emanuele N, Zhang JH, Brophy M, Conner TA, Duckworth W, Leehey DJ, et al. VA NEPHRON-D Investigators: Combined angiotensin inhibition for the treatment of diabetic nephropathy. *N Engl J Med*. 369: 1892–1903, 2013.
24. Unger T. The role of renin-angiotensin system in the development of cardiovascular disease. *Am J Cardiol*. 89(Suppla):3A–10, 2002.
25. Agarwal A, Williams GH,Fisher ND. Genetics of human hypertension. *Trends Endocrinol Metab*. 16:127–133, 2005.
26. SPRINT Research Group, Wright JT Jr., Williamson JD, Whelton PK, Snyder JK, Sink KM, Rocco MV, et al. A randomized trial of intensive versus standard blood-pressure control. *N Engl J Med*. 373: 2103–2116, 2015.
27. Churchill PC, Churchill MC, Bidari AK et al. Genetic susceptibility to hypertension induced renal damage in the rat. *J Clin Invest*. 100:1373–1382, 1997.
28. Toto R. Hypertension in the failing kidney. *Curr Opin Nephrol Hypertens*. 10:165–166, 2001.
29. Krolewski A. Genetic susceptibility to diabetic nephropathy. *Kidney Int*. 55:1582–1596, 1999.
30. Demaine AG, Heesom AE,Cross D et al. Investigation of polymorphisms of aldose reductase promoter region in patients with type 1 diabetes and diabetic microvascular complications. Abstract of 60th *Scientific Sessions of the American Diabetes Association*. San Antonio, Texas, 2000; Abstract 76-OR
31. World Health Statistics World Health Statistics. *Geneva: Global report on diabetes*. 2016.

© 2020 The Authors

https://doi.org/10.46389/rjd-2020-1059
www.rjdnmd.org

Rom J Diabetes Nutr Metab Dis
2020; volume 27, issue 4, pages 410-418

Review

Emerging evidence on the association between COVID-19 and Type 2 Diabetes

Nasreem Bibi, Bahta Wara, Hana Morrissey*, Patrick Ball

School of Pharmacy, University of Wolverhampton, Wolverhampton, WV1 1LY, United Kingdom

*Correspondence to: Hana Morrissey, School of Pharmacy, University of Wolverhampton, Wolverhampton, WV1 1LY, United Kingdom, ORCID: https://orcid.org/0000-0001-9752-537X, E-mail: hana.morrissey@wlv.ac.uk

Received: 1 December 2020 / Accepted: 23 December 2020

Abstract

Objective: Published studies demonstrate that diagnosis with Type 2 diabetes (T2DM) places patients at risk of severe symptoms and increased mortality from COVID-19. The literature was reviewed to understand emerging evidence. **Method:** A review of published studies on COVID-19 in patients with diabetes was conducted to identify the needs and optimal practice for the local population diagnosed with diabetes at risk of COVID-19. **Key Findings:** The combined sample was n=18746 where all patients were diagnosed with T2DM and COVID-19. The severity of symptoms was reported in n=7646. Most reported were fever, (32%) cough (26%), and chest tightness (8%). The causes of death were reported in n=3260. The main causes of death were: COVID-19 (76%), acute respiratory distress (5%). Other comorbidities were reported in n= 6968. The most reported comorbidities were hypertension (38%), cardiovascular (10%), and pulmonary disease (3%). Other risk factors were reported in n= 6968. Those most reported were diabetes, (80%) cardiovascular abnormalities (10%), hyperglycemia not previously diagnosed as diabetes (9%). The reported effects of antidiabetic medications on COVID-19 disease were reviewed for emerging evidence. **Conclusions:** Published studies underline the importance of maintaining weight, glycemic control, good hydration, and exercising as much as possible. Patients need to be informed to present to hospital promptly if developing COVID-19 symptoms. Normal T2DM therapy can be maintained in patients with no, or mild, symptoms. On presentation to hospital with severe COVID-19 disease, diabetes control maybe maintained with insulin, concurrent with hydration and metabolic parameters maintenance until the patient is recovered.

Keywords: Coronavirus, COVID-19, Diabetes Medications, Type 2 Diabetes.

Introduction

The disease caused by the novel coronavirus SARS-Cov-2 (COVID-19) is one of the fastest spreading respiratory system transmitted conditions, and is currently responsible for 13,141,232 cases with 7,660,786 recovered and 573,344 deaths globally [1]. The disease was declared a pandemic, triggering widespread simultaneous lockdowns around the world, with a fluctuating mortality rate around the globe [2]. Currently there is no cure for COVID-19, with a number of pharmacological treatments currently undergoing clinical trials. The virus enters the body via the respiratory system causing a range of symptoms including a high-grade fever, a new or continuous cough, myalgia, gastrointestinal symptoms, and a loss or change in taste or smell [3]. They range from mild, to severe potentially life-threatening, causing hospitalization with pneumonia, acute respiratory distress syndrome (ARDS), multi-organ failure, and death [4].

Studies have described those with obesity, diabetes, and pre-existing cardiovascular conditions as 'at risk groups' and they were the basis of the recommendations on shielding from the World Health Organisation (WHO) [5]. Type 2 diabetes (T2DM) was one of the conditions classed as a risk factor to experience severe COVID-19 symptoms with a higher death rate especially in patients with poor glycemic control [6].

© 2020 The Authors. Romanian Journal of Diabetes, Nutrition and Metabolic Diseases published by Sanatatea Press Group on behalf of the Romanian Society of Diabetes Nutrition and Metabolic Diseases. This is an open access article under the terms of the Creative Commons Attribution License (CC-BY-NC-ND 3.0).

Method

A literature search was conducted using ScienceDirect®, Google® scholar and PubMed®. The search terms used were <coronavirus>, <diabetes> and <type 2 diabetes>. The search was limited to 2019 and 2020 (Fig 1). A total of 10 studies were analyzed.

All articles were screened and reviewed to fit the criteria to assess the association between T2DM and COVID-19 (Appendix 1).

Owing to the lack of randomized controlled trials, it was not possible to combine data from all studies for meta-analysis, so only a narrative review was possible on the information available (Table 1).

Results

All patients reported in the studies selected had been diagnosed with T2DM and COVID-19; there were four domains selected to measure the studies risk of bias score:

1. The study reported on symptoms severity.
2. The study reported on causes of death.
3. The study reported on one or more other risk factors e.g., hypertension, age, obesity, etc.
4. Diabetes medication continued.

The combined sample was n=18746 where all patients were diagnosed with T2DM and COVID-19. Severity of symptoms was reported in n=7646, and the most reported symptoms were fever, (32%) cough (26%), and chest tightness (8%). Causes of death were reported in n=3260 by Huang et al, [4], Zhu et al [6], and Roncon et al [8]. The main causes of death were: COVID-19 (76%) and acute respiratory distress (ARD) (5%). Other comorbidities were reported in n= 6968 by Huang et al [4], Desai et al [11], and Zhou and Tan [12]. The most reported comorbidities were hypertension (38%), cardiovascular (10%), and pulmonary disease (3%). Other risk factors were reported in n= 6968 by Huang et al [4], Desai et al [11], and Zhou and Tan [12]. The most reported risk factors were diabetes, (80%) cardiovascular abnormalities (10%), hyperglycemia not previously diagnosed as diabetes (9%).

Diabetes medications were continued in n= 3185 and stopped in n= 15561 patients. Some medications used to treat diabetes were reported to contribute to poorer outcomes, if risk mitigation was not considered [15]. The authors concluded that metformin was found to contribute to dehydration leading to lactic acidosis and acute kidney injury. They also concluded that the sodium-glucose-co- transporter 2 inhibitors (e.g., canagliflozin, dapagliflozin, and empagliflozin) should not be initiated or continued during the COVID-19 infections as, in addition to increasing the risk of dehydration and acute kidney injury, they can cause ketoacidosis. Bornstein et al [15] stated that glucagon-like peptide-1 receptor

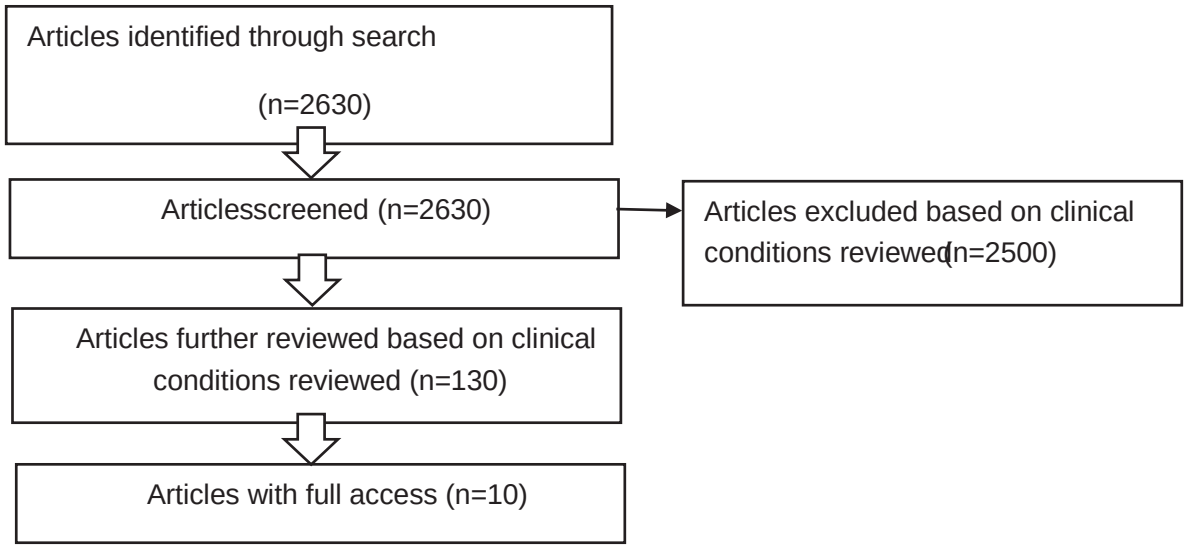

Articles identified through search
(n=2630)
Articlesscreened (n=2630)
Articles excluded based on clinical conditions reviewed (n=2500)
Articles further reviewed based on clinical conditions reviewed (n=130)
Articles with full access (n=10)

Figure 1: Inclusion and exclusion criteria of literature used.

Appendix 1: Final selected papers list (Columns contains a quotation from papers)

Reference	Design	Population	Intervention	Measured outcomes	Results	Conclusion
Zhu et al., 2020[6]	Cohort	7336	Assessing blood glucose levels of T2DM with COVID-19 and	Mortality	Patients had higher mortality (7.8% versus 2.7%: adjusted hazard ratio, 1.49) with multiple organ injury in comparison to those patients that were nondiabetic	Showed patient with improved glycemic control had better outcome with COVID-19 in T2DM patients
Huang et al., 2020[4]	Systematic review	6452	The need for ICU, poor outcome, severe mortality and disease progression	Mortality, severe COVID-19, acute respiratory distress syndrome, ICU and disease progression	T2DM was associated with composite poor outcome (RR2.38 [1.88. 3.03]. p<0.001; I^2 : 62%) and its subgroup which comprised of mortality (RR 2.12[1.44, 3.11], p<0.001; I^2:72%), severe COVID-19 (RR 2.45 [1.79, 3.35], p<0.001: I^2 :45%), ARDS (RR 4.64 [1.86, 11.58], p=0.001; I^2 :9%), and disease progression (RR 3.31 [1.08. 10.14], p=0.04; I^2: 0%	Sever mortality rates were observed in patient with diabetes that were diagnosed with COVID-19
Kumar et al., 2020[7]	Systematic review	114	Assessing studies with patient with COVID-19 in patient hospitalized in ICU	Requiring invasive ventilation, requiring ICU care or progressive disease or refractory disease	Diabetes was found to be significantly associated with mortality of COVID-19 with a pooled odds ratio of 1.90 (95% CI: 1.37e2.64; p <0.01). Diabetes was associated with severe COVID-19 with a pooled odds ratio of 2.75 (95% CI: 2.09e3.62; p<0.01).	Diabetic patient showed to have a 'two-fold increase in mortality as well as severity of COVID-19' compared to those that had no diagnosis of diabetes. Kumar et al., 2020
Roncon et al., 2020[8]	Systematic review	1382	IC admission in T2DM patients-using Newcastle Ottawa quality assessment scale.	To assess the risk of ICU admission and morality risk in diabetic COVID-19 patients.	DM results showed to be the second more frequent comorbidities. DM patients had a significant increased risk of ICU admission (OR: 2.79, 95 % CI 1.85–4.22, p<0.0001, I2=46 %). In 471 patients (mean age 56.6 years, 294 males) analyzed for the secondary outcome diabetic subjects resulted to be at higher mortality risk (OR 3.21, 95 % CI 1.82–5.64, p < 0.0001, I2=16 %).	Diabetic patients with COVID-19 had an increased risk of ICU admission with a higher rate of mortality
Zhang et al., 2020[9]	Cohort	74	To assess HbA_1c in T2DM patients with COVID-19	To describe characteristics of COVID-19 patients with type 2 diabetes and to analyze risk factors for severity	Twenty-seven patients (36.5%) were severe and 10 patients (13.5%) died.	T2DM patient showed to be more susceptible to COVID-19 in comparison to the overall population Zhang et al., 2020

https://doi.org/10.46389/rjd-2020-1059

© 2020 The Authors

Reference	Design	Population	Intervention	Measured outcomes	Results	Conclusion
Raoufi et al., 2020[10]	Cohort	117	CT results, and HbA_1c values were measured to assess severity	Chest CT scores were defined by summation of individual scores from 5 lung lobes	Chest CT severity scores were not significantly different to patient that were T2DM with COVID-19 in comparison to non-diabetic COVID-19 patients Also, the mortality and recovery rates were similar between the two groups ($p = 0.54$ and $p = 0.85$, respectively)	Chest CT severity scores were not significantly different to patient that were T2DM with Corvid-19 in comparison to non-diabetic COVID-19 patients
Desai et al., 2020[11]	Systematic review	2084	Assess COVID -19 symptoms in elderly patients	Mortality rate	T2DM and corvid 19 patient with a mean age >50 years was 13.2% whereas other studies with relatively younger patients mean age <50 years had a pooled prevalence of 9.0%	The overall prevalence of COVID-19 was shown to be higher in elderly patient with T2DM in comparison to the younger population
Zhou and Tan 2020[12]	Cohort	881	Patient capillary blood glucose were taken to assess severity	Mortality	56.6% (499/881) of the tests showed abnormal BG levels, including 29.4% (58/197) of the pre-prandial BG tests and 64.5% (441/ 684) of the postprandial tests. 69.0% (20/29) patients were considered with non-ideal BG levels. And 10.3% (3/29) of the patients suffered at least one episode of hypoglycemia (b3.9 mmol/L).	T2DM is seen to be a co-comorbidity causing severe symptoms in COVID-19
Guo et al.2020[13]	Cohort	174	To assess if T2DM is a risk factor of COVID-19	C- reactive protein test and D-dimer test were used to assess severity in patients	COVID-19 patient without co-morbidities but T2Dm were at higher risk of severe pneumonia, release of tissue injury related enzymes due to dysregulation of glucose metabolism	T2DM should be considered as a risk factor for a rapid progression and deterioration in symptoms in patients with COVID-19
Wang et al 2020[14]	Cohort	132	Bloods were monitored to assess severity of corvid 19	HbA_1c was monitored in patient alongside SaO^2 Mortality rate was also assessed	Correlation analysis showed that there was a linear negative correlation between SaO_2 and HbA_1c ($r = -0.22$, $P = 0.01$), while there was a linear positive correlation between serum ferritin, CRP, Fbg, and ESR levels and HbA1c ($p<0.05$).	High level of HbA_1c is said to cause inflammation and hyper coagulation and low saturation O^2 in COVID-19 patients and the mortality rate 27.7% higher in patient with diabetes

© 2020 The Authors

Table 1: Risk of bias score

Reference	The study reported on symptoms severity	The study reported on causes of death	The study reported on one or more other risk factors	Diabetes medication continued or stopped	Total score	Inclusion	Exclusion
Zhu et al. 2020[6]	Yes	No	Yes	Continued	3	T2DM and COVID-19, between 18 and 75 years of age	No electronic record, pregnant, has organ injury or cancers, laboratory results not available
Huang., et al. 2020[4]	Yes	No	Yes	Not clear	2	T2DM and COVID-19 and need for ICU	Other co-morbidities
Kumar et al. 2020[7]	Yes	Yes	No	Not clear	2	T2DM and COVID-19	Not reported
Roncon et al. 2020[8]	Yes	Yes	Yes	Not clear	3	T2DM and COVID-19 and need for ICU	Not reported
Guo et al. 2020[13]	Yes	Yes	Yes	Continued	4	T2DM and COVID-19	Not reported
Raoufi et al. 2020[10]	No	No	Yes	Continued	2	T2DM and COVID-19	Not reported
Wang et al. 2020[14]	Yes	No	Yes	Not clear	2	T2DM and COVID-19	Not reported
Desai et al. 2020[11]	No	No	Yes	Continued	2	T2DM and COVID-19	Not reported
Zhang et al. 2020[9]	Yes	Yes	Yes	Not clear	3	T2DM and COVID-19	Not reported
Zhou and Tan 2020[12]	No	No	No	Continued	1	T2DM and COVID-19	Not reported

agonists (e.g., insulin glargine with lixsenatide, dulaglutide, exenatide-extended release, liraglutide, lixisenatide, and semaglutide) can be continued subject to monitoring and correcting dehydration. Dipeptidyl peptidase-4 inhibitors (e.g., alogliptin, linagliptin, saxagliptin, and sitagliptin) were generally well tolerated and could be continued [15]. Lastly, they reported that insulin was found to be the most beneficial and best tolerated, and should always be continued.

Narrative analysis

Zhu et al [6] examined the association between glycemic control and outcome in patients with COVID-19 and pre-existing T2DM. In their retrospective multi-center study, they concluded that T2DM is a major co-morbidity of COVID-19. Zhu et al [6] explained that glycemic control in T2DM is an important factor in how an individual is impacted with COVID-19 and when poor, increased the likelihood of a severe presentation compared to those with no diabetes. Their study also revealed that T2DM patient required more time in intensive care units compared to non-diabetic patients. A gender difference was identified with males with T2DM at higher risk than females. Paland Bhadada [2] discussed the interaction between the two diseases and reviewed how T2DM placed individuals at a disadvantage. They found a clear relationship between

https://doi.org/10.46389/rjd-2020-1059
© 2020 The Authors

the two conditions and on how they combine to precipitate a severe outcome in response to the COVID-19 infection. They hypothesized that the interaction occurs because of the "*exagerated pro-inflammatory cytokine response and low expression of angiotensin-converting enzyme 2 (ACE2)*" enhancing the severity of the symptoms caused by the virus. They also explain how SARS-Cov-2 causes worsening glycemic control. This is postulated to be due to SARS-Cov-2 mediated damage to pancreatic beta cells causing "*augementation of insulin resistance through cytokines and ferritin A and hypokalemia*" [2]. The study by Singh and Singh [16] supported Banerjeeh et al [17] who focused on the relationship between poor glycemic control and the increased risk of severe presentation of COVID-19 symptoms.

Huang et al [4] concluded that COVID-19 pneumonia was both more severe and more often fatal in T2DM patients than in non-diabetic patients. They described how other risk factors alongside T2DM such as hypertension, particularly when influenced by age, could further worsen the patient outcomes [4]. Zhang et al [9] also reported that patients with uncontrolled T2DM were more susceptible to COVID-19 infection, with higher mortality and severity of symptoms.

Bouhanick et al [18] stated that "*whilst diabetes would not systemically increase the likelihood of being infected with COVID-19, once infected patients with diabetes are likely to develop a severe form of the disease*". The authors also highlighted the association of T2DM medication and how it may impact the severity in symptoms of COVID-19. They explain how dipeptidylpeptidase-4 inhibitors can "*via the inflammatory effect, play arole in the course of the infection or in the occurrence of its complications*" [18]. The authors highlighted other T2DM medications that should be used with caution or discontinued in patients with COVID-19. These included ipeptidylpeptidase-4 inhibitors, glucagon like peptide-1 agonists, sodium-glucose co-transporter 2 inhibitor, and metformin [18]. Mukherjee et al [19] concluded that "*pioglitazone has more potential for benefit than harm and can be continued in people with T2DM and mild/moderate COVID-19*" unless contraindicated in the specific patient. The pharmacology of the thiazolidinediones is said to play a vital role against COVID-19 through the moderation of host inflammatory responses that are driving hyperinflammation [19].

Bornstein et al [15] explained the nature and complexity of diabetes and how it is generally a primary risk factor for severe pneumonia in up to 50% in a diabetic patients compared to non-diabetic patients. The increased risk of infection is postulated to be due to "*defects in innate immunity affecting phagocytosis, neutrophil chemotaxis and cell mediated immunity*" [15]. In a meta-analysis, Kumar et al [7] described a strong correlation between diabetes and severe COVID-19 symptoms, from the pooled data of 16,003 patients, leading to a two-fold increase in mortality and severity of COVID-19 as compared to non-diabetics. Roncon et al [8] further explained that the diabetic patient is at higher risk of ICU admission, poor short-term outcome, and poor survival rate due to exhibiting deteriorating, disabling, severe symptoms. Raoufi et al [10] demonstrated that the chest computed tomography score did not show a statistically significant difference between the two groups.

Age is reported to impact outcome. Desai et al [11] pooled data on various age groups to understand the effect on patients with COVID-19 and T2DM. Their results revealed older patients showed an increased chance both of contracting COVID-19 and experiencing greater severity of symptoms. They reported that a patient, mean age <50 had a 9% chance of contracting COVID-19 compared to 13.2% for >50 years of age. They also considered comorbidities such as cardiovascular complications, and reported that increasing comorbidities alongside age may further impact T2DM / COVID-19 patients. The retrospective study of Guo et al [13] (n=174) found patients with T2DM were at a higher risk of severe pneumonia compared to patients without other comorbidities. Patients were screened with chest computed tomography (CT) alongside other treatment measures. They determined that 24 of their 174 patients were at higher risk of pneumonia and "*excessive uncontrolled inflammatory responses*" [13]. They concluded such patients required closer attention and were to be reviewed

© 2020 The Authors

accordingly due to their potential for rapid deterioration.

Zhou and Tan [12] reviewed the records of 881 patients concluding that diabetes and hyperglycemia can lead to higher secondary infection risks and mortality and therefore, tight glycemic control is essential in COVID-19 patients. Wang et al [14], in a retrospective study of 132 patients, found the mortality rate was higher in patients with T2DM by 27.7% and that high HbA_1c levels were associated with inflammation, hypercoagulability, and low oxygen saturations. They emphasized the importance of measuring HbA_1c levels post hospital admission to improve the prognosis of COVID-19 [14]. They also explained how COVID-19 potentially caused abnormal beta cell damage and insulin resistance causing blood glucose levels above their baseline in all patients.

Antidiabetic medication and COVID-19

Medications used to treat diabetes can lead to further deterioration in COVID-19 due to dehydration and renal injury. Banerjee et al [17] highlighted how the pandemic may have had an impact on glycemic control; reduced physical activity, more chance of eating at home and of consuming high energy take-away food affecting weight and consequently their diabetes [17].

Bouhanick et al [18] reviewed the pharmacological characteristics of T2DM treatment and the linkage to their effect on COVID-19. They discussed dipeptidyl peptidase-4 (DPP-4) inhibitors and the potential increased risk of infection through stimulating an 'inflammatory immune response through modifying the production of several cytokines and chemokines' [18]. They noted that other studies agreed that DPP-4 inhibitors in T2DM patients, particularly sitagliptin, increased the risk of upper respiratory tract and lower urinary tract infections.

Nakhleh and Shehadeh [20] agreed that T2DM medications may have deleterious effects on patients presenting with COVID-19. However, they also noted how some T2DM treatment may offer a protective role in COVID-19 related lung injury [20]. Insulin is an effective treatment to continue in diabetic patients positive for COVID-19 as it inhibits the production of pro-inflammatory factors preventing acute lung injury. It also reduces the risk of developing diabetic ketoacidosis or hyperglycemic hyperosmolar states preventing further deterioration of any acute lung injury and acute respiratory distress [20].

Bornstein et al [15] stated that biguanides and SGLT-2 inhibitors should be withheld in patients with severe symptoms of COVID-19 to prevent the risk of metabolic decompensation [15]. They recommend that after cessation, insulin should be used for acute control. Conversely, regarding the DDP-4 inhibitors, Bornstein et al [15] differed from Bouhanick et al [18] by concluding they believed there was no convincing evidence to suggest DDP-4 inhibitors should be discontinued.

Ursini et al [21] focused on biguanides in COVID-19 patients. Their article on "COVID-19 and diabetes: Is metformin a friend or foe" describes how metformin and other medications such as pioglitazone and liraglutide may play a role in promoting the SARS-Cov-2 virus entry into host cells, they are said to 'work synergistically to increase the angiotensin converting enzyme-2 availability in the respiratory tract promoting SARS-Cov-2 infection' [21]. On the other hand, the author expounds how these medications may be advantageous in maintaining the optimal management of glycemic control and therefore need to be monitored to assess whether they are beneficial to be continued in patients who are positive COVID-19 [21].

A scoping review by Wicaksana et al [22] focused on the pharmacological management of T2DM patients during the pandemic. They also noted the importance of glycemic control and the prevention of hypoglycemia. They advised that adjustment of 'sulfonylurea and insulin dose may be necessary to prevent hypoglycemia' and should be handled with caution in elderly patients as they are more likely to have deteriorating glycemic control. They also explained the importance of close glycemic control during the COVID-19 pandemic due to the restrictions imposed; the social distancing and lockdown affecting individual weight, health, and mental state causing poorer glycemic control, and

https://doi.org/10.46389/rjd-2020-1059
© 2020 The Authors

pressure on staff in hospitals overloaded with Covid admissions [22].

Singh and Khunti [23] presented a concise table on anti-diabetic medications that may have a deleterious effect on patients diagnosed with COVID-19. The table showed that Metformin, Pioglitazone, Sulfonylurea, DPP-4 inhibitors, SGLT-2 inhibitors, and GLP-1 receptor agonists can all be continued in patients with mild to moderate COVID-19 disease but should be withheld in those presenting with severe symptoms, whilst insulin may be continued at any stage [23].

Mukherjee et al [19] analyzed the use of pioglitazone in patients with COVID-19. They explained how pioglitazone had potential for more benefit than harm and could be continued as advised also for mild/moderate COVID-19 disease.

It appears clear that tight glycemic control appears advantageous, but must be weighed against the risk of precipitating hypoglycemic episodes.

Limitations

COVID-19 is an extremely fast-moving field, and it is important to try to learn lessons as they emerge. However, most of the studies reported collected data in the early phase of the epidemic when many health services were overloaded and under pressure, whilst emerging data was constantly modifying policies and practices. It quickly became clear that T2DM is a major risk factor for severe presentations of COVID-19, and it is important that pharmacists learn the lessons of early experience in relation to diabetes and anti-diabetic drugs. This review presents and summarizes the information available at the time of submission.

Conclusion

Managing COVID-19 disease continues to follow emerging evidence which appears almost daily, but it is essential to pause and try to review the best information available to date in order to adapt and try to ensure our management reflect the current evidence. Clearly diabetes and particularly T2DM are risk factors for worsening prognosis in COVID-19. Based upon the above we believe the current understanding of the evidence is:

1. Patients diagnosed with T2DM in lockdown need to the best of their ability, to maintain good weight, good glycemic control, good hydration, and remain physically active.
2. Practitioners should provide information on the importance of early presentation to healthcare providers if COVID-19 symptoms are developing.
3. Following the treating practitioner's directions where possible, but taking note of the findings from previous studies. This suggests most patients with no symptoms or only mild symptoms of COVID-19 can maintain their normal therapy in T2DM patients.
4. Patients with severe COVID-19 symptoms, on presentation to hospital, most probably should have their oral therapy withheld and replaced by insulin while hydration and metabolic parameters are monitored until the patient has recovered.
5. This, like all other aspects of COVID-19 management, remains a fast-moving field and advice may change as more information emerges.

Conflict of interest

The authors declare no conflict of interest.

Funding

No external funding.

Ethics clearance

This is a review article based on data already published in other research and does not include any new real life data; so it is exempted under the university and the UK health research approval requirement.

© 2020 The Authors

Authors' contributions

The 1st and 2nd authors conducted the review, 3rd and 4th authors completed the paper.

References

1. World meter website. COVID-19 Coronavirus Pandemic. Available at: https://www.worldometers.info/coronavirus/. Accessed 09/07/20.
2. Pal R, Bhadada SK. COVID-19 and diabetes mellitus: An unholy interaction of two pandemics. *Diabetes Metab Syndr*. 14(4):513–517, 2020.
3. National Health Service. Check if you or your child has Coronavirus symptoms. Available at: https://www.nhs.uk/conditions/coronavirus-covid-19/symptoms/. Accessed 09/07/20.
4. Huang I, Lim MA, Pranata R. Diabetes mellitus is associated with increased mortality and severity of disease in COVID-19 pneumonia – A systematic review, meta-analysis, and meta-regression. *Diabetes Metab Syndr*. 14(4):395–403, 2020.
5. European Centre for Disease Prevention and Control. COVID-19 situation update worldwide. Available at: https://www.ecdc.europa.eu/en/geographical-distribution-2019-ncov-cases. Accessed 13/07/20.
6. Zhu L, She ZG, Cheng X, Qin JJ, Zhang XJ, Cai J, et al. Association of Blood Glucose Control and Outcomes in Patients with COVID-19 and Pre-existing Type 2 Diabetes. *Cell Metab*. 2;31(6):1068–1077.e3, 2020.
7. Kumar A, Arora A, Sharma P, Anikhindi SA, Bansal N, Singla V, et al. Is diabetes mellitus associated with mortality and severity of COVID-19? A meta-analysis. *Diabetes Metab Syndr*. 14(4):535–545, 2020.
8. Roncona L, Zuinb M, Rigatellic G, Zulianib G. Diabetic patients with COVID-19 infection are at higher risk of ICU admission and poor short-term outcome. *J Clin Virol*. 127:104354, 2020.
9. Zhang Q, Wei Y, Chen M, Wan Q, Chen X. Clinical analysis of risk factors for severe COVID-19 patients with type 2 diabetes. *J Diabetes Complications*. 2020;34(10):107666. doi:10.1016/j.jdiacomp.2020.107666.
10. Raoufi M, Khalili S, Mansouri M, Mahdavi A, Khalili N. Well-controlled vs poorly-controlled diabetes in patients with COVID-19: Are there any differences in outcomes and imaging findings? *Diabetes Res Clin Pract*. 166:108286, 2020.
11. Desai R, Singh S, Parekh T, Sachdeva S, Sachdeva R, Kumar G. COVID-19 and diabetes mellitus: A need for prudence in elderly patients from a pooled analysis. *Diabetes Metabol Syndr*. 14(4):683–685, 2020.
12. Zhou J, Tan J. Diabetes patients with COVID-19 need better blood glucose management in Wuhan, China. *Metabolism*. 107:154–216, 2020.
13. Guo W, Li M, Dong Y, Zhou H, Zhang Z, Tian C et al. Diabetes is a risk factor for the progression and prognosis of COVID-19. *Diabetes Metab Res Rev*. 3319, 2020.
14. Wang Z, Du Z, Zhu F. Glycosylated hemoglobin is associated with systemic inflammation, hypercoagulability, and prognosis of COVID-19 patients. *Diabetes Res Clin Pract*. 164:108214, 2020.
15. Bornstein SR, Rubino F, Khunti K, Mingrone G, Hopkins D, Birkenfeld AL, et al. Practical recommendations for the management of diabetes in patients with COVID-19. *Lancet Diabetes Endocrinol*. 8(6):546–550, 2020.
16. Singh AK, Singh R. Does poor glucose control increase the severity and mortality in patients with diabetes and COVID-19? *Diabetes Metabol Syndr*. 14(5):725–727, 2020.
17. Banerjee M, Chakraborty S, Pal P. Diabetes self-management amid COVID-19 pandemic. *Diabetes Metabol Syndr*. 14(4):351–354, 2020.
18. Bouhanick B, Cracowski JL, Faillie JL; French Society of Pharmacology, Therapeutics (SFPT). Diabetes and COVID-19. *Therapie*. 2020;75(4):327-333. doi:10.1016/j.therap.2020.05.006.
19. Mukherjee JJ, Gangopadhyay KK, Ray S. Use of pioglitazone in people with type 2 diabetes mellitus with coronavirus disease 2019 (COVID-19): Boon or bane? *Diabetes Metabol Synd*. 14(5):829–831, 2020.
20. Nakhleh A, Shehadeh N. Interactions between antihyperglycemic drugs and the renin-angiotensin system: Putative roles in COVID-19. A mini-review. *Diabetes Metabol Synd*. 14(4):509–512, 2020.
21. Ursini F, Ciaffi J, Landini MP, Meliconi R. COVID-19 and diabetes: Is metformin a friend or foe? *Diabetes Res Clin Pract*. 164:108–167, 2020.
22. Wicaksana AL, Hertanti NS, Ferdiana A, Pramono RB. Diabetes management and specific considerations for patients with diabetes during coronavirus diseases pandemic: A scoping review. *Diabetes Metabol Syndr*. 14(5):1109–1120, 2020.
23. Singh AK, Khunti K. Assessment of risk, severity, mortality, glycemic control and antidiabetic agents in patients with diabetes and COVID-19: A narrative review. *Diabetes Res Clin Pract*. 165:108–266, 2020.

https://doi.org/10.46389/rjd-2020-1059
© 2020 The Authors

https://doi.org/10.46389/rjd-2020-1060
www.rjdnmd.org

Rom J Diabetes Nutr Metab Dis
2020; volume 27, issue 4, pages 419-430

Review

Dietary acid load: focus on body pH homeostasis and drug responses in type 2 diabetes

Farid Berroukeche[1,2]*, Ismail Belkadi[2], Ouiam Halhali[2], Nassima Mokhtari-Soulimane[1], Hafida Merzouk[1]

[1] Laboratory of Physiology, Pathophysiology and Biochemistry of Nutrition, Department of Biology, Faculty of Natural and Life Sciences, Earth and Universe, University of Tlemcen 13000, Algeria
[2] Faculty of Medecine, University of Bechar, B.P 417 Kenadsa road, Bechar 08000-Algeria

*Correspondence to: Farid Berroukeche, Street 95, N° 01 Emir AEK Street, Maghnia, Tlemcen, Algeria. E-mail: f.berroukeche@gmail.com, Phone: +00 213 662 617 077

Received: 6 May 2020 / Accepted: 24 July 2020

Abstract

Diabetes mellitus is a heterogeneous group of metabolic dysregulation that shares phenotype of hyperglycemia and for which genetic and environmental risk factors act synergistically. Dietary acid load and low pH of interstitial fluid are the most important, factors reducing insulin sensitivity and making the body condition worse in Type 2 diabetes mellitus (T2DM). Several pharmacological classes of oral antidiabetic drugs (OADs) are actually reachable for the treatment of T2DM. These drugs are designed especially to reduce blood glucose level but not necessary to improve insulin resistance. This latter with OADs side effects may affect the whole therapeutic strategies of T2DM. The kind of diet can deeply affect the organism by the generation of acid or base precursors. Indeed, foods such as meat, eggs, cheese, and grains increase the production of acid in the organism, while fruit and vegetables are alkalizing. However, milk, fats and sugars are considered neutral, which have an insignificant effect on acid–base balance. To save cell function, the pH of body fluids is maintained constant by various systems which became impaired with increase in age and many pathological situations. This review proposes to highlight the effect of dietary acid load on the pathogenesis and management of T2DM as well as on its influence on the heterogeneity of OADs responses observed in diabetic patients.

Keywords: Diet acid–base load, Diabetes, Potential renal acid load (PRAL), Insulin resistance, drug response.

Introduction

Diabetes is a pandemic non-infectious disease. It is a leading worldwide cause of mortality and disability [1]. The international prevalence data report that in 2017, 425 million people were diagnosed as suffering from diabetes mellitus worldwide, and the number of people with diabetes mellitus is considered to rise up to 629 million by 2045 [2]. However, more than 90% of these patients are type 2 diabetes (T2DM) [3]. The most important symptoms of T2DM are hypoglycemia, which is complicated by insulin resistance resulting from insulin hypersecretion caused by pancreatic β-cell dysfunction [4]. Chronic hyperglycemia results from glucose metabolism impairment in cells, such as hepatocytes, adipocytes, skeletal muscles, etc., frequently leading to irreversible complications like: macro-and micro-vascular complications, myocardial stroke, renal failure, peripheral neuropathy and blindness [5]. Numerous researchers are reached to develop various types of glucose lowering drugs for treatment of T2DM such as sulfonylurea, glucosidase inhibitors, biguanide, thiazolidine, dipeptidyl-peptidase (PPD) IV inhibitors, sodium-glucose cotransporter 2 (SGLT2) inhibitors [6]. Nevertheless, an extremely large number of people are still suffering from T2DM. This means that the above mentioned drugs are still effective from a viewpoint of reduction of blood

© 2020 The Authors. Romanian Journal of Diabetes, Nutrition and Metabolic Diseases published by Sanatatea Press Group on behalf of the Romanian Society of Diabetes Nutrition and Metabolic Diseases. This is an open access article under the terms of the Creative Commons Attribution License (CC-BY-NC-ND 3.0).

glucose level, but not on fully treated patients suffering from T2DM with insulin resistance [5]. Several publications have appeared in recent years documenting the implication of dietary acid load in the increasing risk of T2DM through insulin resistance [6–9]. In addition, Puddu et al. suggest that weak short-chain fatty acids (weak organic acids) produced by bacterial fermentation in distal gut may stimulate the secretion of glucagon-like peptide 1 (GLP-1) that improves insulin resistance; however, the exact molecular mechanism is still unclear [10].

Interstitial fluids provide a biochemical medium for extracellular signaling molecules such as neurotransmitters and hormones (insulin) to regulate cell function. However, variation of interstitial space composition disturbs efficiency signal transduction of this signaling molecule from extracellular cells domain to intracellular effective signal. Presence of weak pH buffering molecules in interstitial fluids makes pH homeostasis of this extracellular medium vulnerable to proton load [5]. Under anerobic conditions, glucose metabolism (glycolysis and glycogenolysis) generates protons from lactic acid (lactate−/H^+) in adipose and skeletal muscle tissues [11]. Ketone bodies (β-hydroxybutyrate−/H^+) are other major sources of protons of fatty acid in the liver cells [6, 12]. In contrast to interstitial space, blood has a powerful pH buffering such as albumin, hemoglobin, which keep the rigorous pH of blood at a range between 7.35 and 7.45. These facts mean that even if the pH of the blood stays at normal value, pH of interstitial space would deviate from the physiological range under the effects of pathophysiological metabolic conditions [5]. Disruption of pH homeostasis in intra or extra cellular space has many consequences in cells metabolism or tissues affecting mainly pH-sensitive membrane transporters, metabolic enzymes (phosphofructokinase) and hormones (insulin-receptor binding) [13].

Effective solutions are needed to slow or reverse this situation, especially by acting in variable factors, including physical activity, weight and diet. The role of lifestyle and nutrition in the management and prevention of T2DM is very clear through its effect on body weight and metabolic control [2]. Furthermore, appropriate diet and several bioactive factors involved in regulating organic acid production and proton clearance may be vital for the prevention or improvement of metabolic dysfunction linked with T2DM.

In this review article we try to highlight the influence of alkaline/acid diet load on pH homeostasis of body fluids, insulin resistance, and on drug response of T2DM. Further, this work proposes perspective therapies on the basis of regulation of body fluid pH including diet.

Cells and interstitial pH space homeostasis

Chemical reactions in living organisms are influenced mainly by pH. This latter affects the charge of reactive groups of molecules and enzymes within extracellular and intracellular fluids (Table 1). The chemical activity or concentration of the H^+ or hydronium ion (H_3O^+) is remarkably small and stable, given the abundance of protons in body fluids [14]. Intracellular pH (pHi) is an important constant of cytoplasmic compartment which can touch approximately all aspects of cell functions. pHi changing may affect predominantly: pH-sensitive metabolic enzymes [15], such as phosphofructokinase (a key of glycolytic enzyme), polymerization, and cross-linking of cytoskeletal proteins such as actin and tubulin, the ability of muscle cells to generate tension (muscle weakness), gap junctions, many ion-selective channels, apoptosis, cell growth, and proliferation signal and osmosis. Furthermore, fluctuation of intracellular pH also leads to the change of cell responses to external informational factors, including neurotransmitters, growth factors, and hormones like insulin. For that, all cells are well-equipped with several chemical defense systems to fight against pH fluctuations. Indeed, to maintain a stable pHi, tremendous cellular buffers are implicated to regulate pHi supported by the activity of different categories of membrane bound transporters. These transporters are classified into five types: 1. Proton pumps or H^+-ATPases; 2. Cation/H^+ exchangers, for example the alkalinizing Na^+/H^+ exchanger and the acidifying K^+/H^+ exchanger; 3. Na^+-organic anion co-transporters; 4. Cl^-/organic anion exchangers; and 5. HCO_3^- dependent

https://doi.org/10.46389/rjd-2020-1060
© 2020 The Authors

transporters, such as the (Na^++ HCO_3^-) /Cl^- and the Cl^-/HCO_3^- exchangers and Na^+-HCO_3^- co-transporters [13].

As mentioned above, to maintain the intracellular pH at physiological levels, the acids generated in the intracellular medium is extruded into the extracellular fluid (the interstitial space). The acidity of this latter is suggested to be one of the most serious pathogenesis mechanisms leading to various diseases, including tumor metastasis and diabetes mellitus [8, 9].

However, the organs directly linked to the maintenance of acid base homeostasis are lungs and kidneys, as well as a complex system of buffers. The interaction of these mentioned elements is required to save the arterial pH in physiological ranges (7.35–7.45) [7]. This equilibrium is maintained via the involvement of three interconnected mechanisms: blood and tissue buffering processes (e.g., bicarbonate), the redistribution of carbon dioxide (CO_2) from the blood to the lungs through respiration, and the excretion of excess hydrogen ions from the blood into the urine via kidneys. During every given moment, the acid-base equilibrium is mostly affected by cell metabolism (e.g., exercise), food intake, and disease patterns which it is also considered to affect either acid generation (e.g., diabetic ketoacidosis) or excretion (e.g., renal failure) [16].

Another mechanism is widely involved in acid base balance of the body which we may not ignore. Bone is a very large ion exchange buffer system which contains 99% of body calcium. Furthermore, Barzel and Massey added that 80% of the total body carbonate are in the hydration shell. Then, the water surrounding bone, are 80% of citrate and 35% of sodium, which can serve to buffer the excess acid. Bone replies to acidity by an acellular, physicochemical reaction with the immediate release of carbonate, citrate and sodium from the hydration shell. In response to

Table 1: pH of selected fluids, organs, and cell compartment.

Organ, fluid or intracellular organelle	pH	References
Skin	Natural pH is between 4 and 6.5	[16]
Urine	4.6–8	[16]
Gastric	1.35–3.5	[16]
Bile	7.6–8.8	[16]
Pancreatic Fluid	8.8	[16]
Vaginal Fluid	<4.7	[16]
Cerebro-spinal Fluid	7.3	[16]
Serum venous	7.35	[16]
Serum arterial	7.4	[16]
Breast milk	6.35–7.35	[19]
Intracellular Fluid	6.0–7.2	[16]
Cis Golgi apparatus	6.7	[20]
Medial Golgi apparatus	6.3	[20]
Trans Golgi apparatus	6.0	[20]
Secretory vesicles	5.5	[20]
Early endosome	6.5	[20]
Late endosome	6	[20]
Lysosome	<5.5	[20]
Mitochondria	7.5–8.0	[13]

© 2020 The Authors

chronic acid stress, such as imposed by an acid-ash diet, cellular responses mobilize bone and calcium as a buffer. An acid-ash diet is a diet that creates acid in the process of its metabolisms [17].

Therefore, under physiological conditions (anaerobic stress) a little amount of pyruvate generated by glycolysis is converted to lactate. However, the rest of pyruvate is transformed in the mitochondrial tricarboxylic acid cycle (TCA) to produce CO_2 (major sources for H^+), which is converted later into H^+ and HCO_3^- by carbonic anhydrase. Further, in a similar situation, to produce the same amount of ATP, glycolysis pathway generates more protons than the both metabolic ways (glycolysis and TCA cycle) in normal condition. This explains that glycolysis associated with mitochondrial TCA cycle impairment is more acidogenic than glycolysis followed with full functional TCA cycle. Previous studies also indicated a reduction of mitochondria function in persons with T2DM [5, 18] where the total amount of H^+ produced in these patients is much larger than that in healthy subjects with normal mitochondrial function [18].

Dietary and acid load Metabolism

Food is one of the main determinants of endogenous acid production [5]. It defines the formation of acids, alkalis or neutral biochemical species once absorbed and metabolized. Main nutrient components liberating acid precursors are presented by phosphorus and proteins (mostly the sulfur amino acids such as cysteine, taurine, and methionine, in addition to cationic amino acids like arginine and lysine). However, some minerals such as potassium, magnesium and calcium are considered to be an alkaline precursor [7].

From a practical point, the main so-called "acidifiers" foods are generally of animal origin: meat, fish, poultry, eggs, and shellfish. However, some proteins of plant origin, such as those from cereals and legumes, containing many sulfur amino acids, have also a strong acidifying power. Further, milk, by its high content of calcium (alkalizing), is considered neutral. Sugar and lipids have a weak acidifying power and a little contribution in acid-base balance [21]. Conversely, the alkalizing foods are mainly the fresh fruits and vegetables, which are particularly rich in magnesium and potassium. The alkalizing power of food is in fact appreciated by its potassium content [22]. So, the potatoes and pumpkins, are rich in K^+, and have a greater anti-acidifying effect than apples and pears. In practice, the acidifying or basifying power of diet can be approached by two methods [23]:

The First PRAL (potential renal Acid load) score was estimated using an algorithm described by Remer and colleagues [24]: PRAL (mEq/d) = 0.4888 x protein intake (g/day) + 0.0366 x phosphorus (mg/day) – 0.0205 x potassium (mg/ day) – 0.0125 x calcium (mg/day) – 0.0263 x Magnesium (mg/day). However, the NEAP (net endogenous acid load) score was calculated using the formula described by Frassetto et al., [25]: estimated NEAP (mEq/d) = [54.5 x protein intake (g/day) ÷ potassium intake (mEq/day)] – 10.2.

The values of the first equation describe the acid or alkaline load produced by each food, it is expressed by mEq/100 g. However, the second formula result from the acidifying effect of sulfuric amino acids such as cysteine and methionine contained in protein food and alkalizing action of the mineral salts, anions of weak organic acids and cationic amino acids mainly present in vegetable foods [7, 26].

The calculated PRAL and NEAP scores obtained using the above equations were well evaluated against both the PRAL and NEAP scores assessed from 24-hour urine collections. In fact, when the PRAL value for a type of food is less than 0, it is considered that this food enhances the pH of body fluids (alkalinity) and, when it is more than 0, that food tends to increase the production of acids in the body (acid load). Usually, foods such as eggs, meat, cheese and grains increase the production of acids in the body, while fruit and vegetables are alkalizing diets [22] (Table 2). The amount of discharged acid of proteins is widely linked to the chemical nature and the ways which the amino acids are metabolized. Indeed, some of these are categorized as neutral, others as acidic, and certain as alkaline. For example, histidine, lysine, and arginine, are considered acidic when metabolized, they generate hydrochloric acid, just

https://doi.org/10.46389/rjd-2020-1060
© 2020 The Authors

Table 2: Nutrient content and estimated potential, renal acid load of frequently consumed foods and beverages (related to 100 g or ml of edible portion) [28].

Food or food group	PRAL (mEq) 100 g or mL
Beverages	
Coca-Cola	0.4
Coffee, infusion, 5 minutes	–1.4
Mineral Water (Volvic)	–0.1
Mineral Water (Apollinaris)	–1.8
Tea, Indian, infusion	–0.3
Red wine	–2.4
White wine, Dry	–1.2
Fats and Oils	
Butter	0.6
Margarine	–0.5
Olive oil	0.0
Sunflower Seed oil	0.0
Fish	
Cod, fillet	7.1
Haddock	6.8
Trout, brown, steamed	10.8
Fruits, nuts, and fruit juices	
Apple juice, unsweetened	–2.2
Apricots	–4.8
Bananas	5.5
Cherries	3.6
Grape juice, unsweetened	1.0
Kiwi fruit	–4.1
Lemon juice	–4.1
Lemon juice	–2.5
Orange juice, unsweetened	–2.9
Orange	–2.7
Peaches	–2.4
Peanuts, plain	8.3
Raisins	–21.0
Strawberries	–2.2
Walnuts	6.8
Water melon	–1.9
Grain products	
Bread, rye flour, mixed	4.0
Bread rye flour	4.1

Table 2: Coninued

Food or food group	PRAL (mEq) 100 g or mL
Bread, wheat flour, whole meat	1.8
Bread, white wheat	3.7
Rice, brown	1.5
Rice, white, easy cook	4.6
Rice, white, easy cook, boiled	1.7
Spaghetti, white	6.5
Spaghetti, whole meal	7.3
Wheat flour, white, plain	6.9
Wheat flour, whole meal	8.2
Legumes	
Beans, green/french beans	–3.1
Lentils, green and brown, whole, dried	3.5
Peas	1.2
Meat and meat product	
Beef, lean only	7.8
Chicken, meat only	8.7
Corned beef, canned	13.2
Liver sausage	10.6
Rump steak, lean and fat	8.8
Turkey, meat only	9.9
Veal, fillet	9.0
Milk, dairy products, and eggs	
Buttermilk	0.5
Camembert	14.6
Cheddar-type, reduced fat	26.4
Cheese, Gouda	18.6
Creams, fresh, sour	1.2
Eggs, chicken, whole	8.2
Eggs, yolk	23.4
Milk, whole evaporated	1.1
Milk, whole pasteurized and sterilized	0.7
Yogurt, whole milk, fruit	1.2
Yogurt, whole milk, plain	1.5
Sugar, and sweets	
Chocolates, milk	2.4
Honey	–0.3

© 2020 The Authors

Table 2: Coninued

Food or food group	PRAL (mEq) 100 g or mL
Sugar, white	–0.1
Vegetables	
Asparagus	–0.4
Broccoli, green	–1.2
Carrots, young	–4.9
Cauliflower	–4.0
Celery	–5.2
Cucumber	–0.8
Leeks	–1.8
Lettuce, average of 4 varieties	–2.5
Mushrooms, common	–1.4
Onions	–1.5
Peppers, capsicum, green	–1.4
Potatoes, old	–4.0
Radish, red	–3.7
Spinach	–14.0
Tomatoes	–3.1
Zucchini	–4.6

as methionine and cysteine, which contain sulfur and are transformed into sulfuric acid [22, 27]. Furthermore, the alkaline nutrients are generally organic acids precursors such as citrate or bicarbonate releasers during their catabolism [23].

Cross link between Diet acid load balance and Diabetes

As mentioned above, the endogenous acid production is mainly dependent on the metabolism of food and widely varies with its nature. The dietary acid load is eliminated by the functionally normal kidney, which thus maintains the acid-base balance. However this function of healthy kidneys becomes impaired with the increase in age. Maintaining acid-base homeostasis are ensured by neutralization of the number of milli-equivalent of non-carbonic and non-volatile acids produced with an equal amount of protons before excretion through the urine [26]. Data found that urine pH also fell with age, and sex. In fact, women are known to produce alkaline urine compared to men at all ages. Obesity and diabetes had a lower urinary pH when the prevalence of both increases with age [29]. For that, the elderly people frequently develop chronic low grade metabolic acidosis, linked to lower retention of filtrate bicarbonates, and a reduction of acid excretion, which results in a decrease in capacity response to acid charge compared to younger subjects. This low-grade metabolic acidosis, which progresses with age, is characterized by a lower limit of normal pH and plasma bicarbonates value despite a positive balance sheet of H^+ ions [30]. Chauveau et al. [26] demonstrated that a chronical uptake of high acid load food could accentuate the development of this acidosis. They added that the ingestion of alkaline diet rich in potassium salts, provided by fruits and vegetables, is able to prevent the low grade muscular metabolic acidosis observed in the elderly subjects [26] and reduces risk of developing T2DM [31].

Nevertheless, metabolic acidosis is also associated with a defect of insulin-sensitivity and an increase in the prevalence of glucose intolerance [32]. The underlying mechanisms would likely result from insulin failure to link to its peripheral receptor in interstitial space, as well as a disruption of the intracellular PI3K signalling pathway by acidosis, physiologically observed in the downstream of insulin stimulation (Fig. 1) [23].

Vegetarians have significantly lower rates of developing T2DM than do omnivores. This may be partly explained by the greater BMI of omnivores compared with vegetarians [33]. In fact, vegetable-based diets are rich in soluble fiber and low glycemic index of carbohydrates (legumes, whole grain products such as oats and barley, fruit and vegetables) characterized by slow intestinal absorption and minimal postprandial insulin secretion, avoiding hyperinsulinemia and insulin resistance. Plant-based diets also demonstrate favorable metabolic effects in other populations [31]. However, meat and processed meat intake alone was found to be an important risk factor for diabetes even after adjustment for BMI. Higher intakes of plant foods, such as vegetables, whole grain foods, legumes, and nuts, but not fruit juice,

https://doi.org/10.46389/rjd-2020-1060
© 2020 The Authors

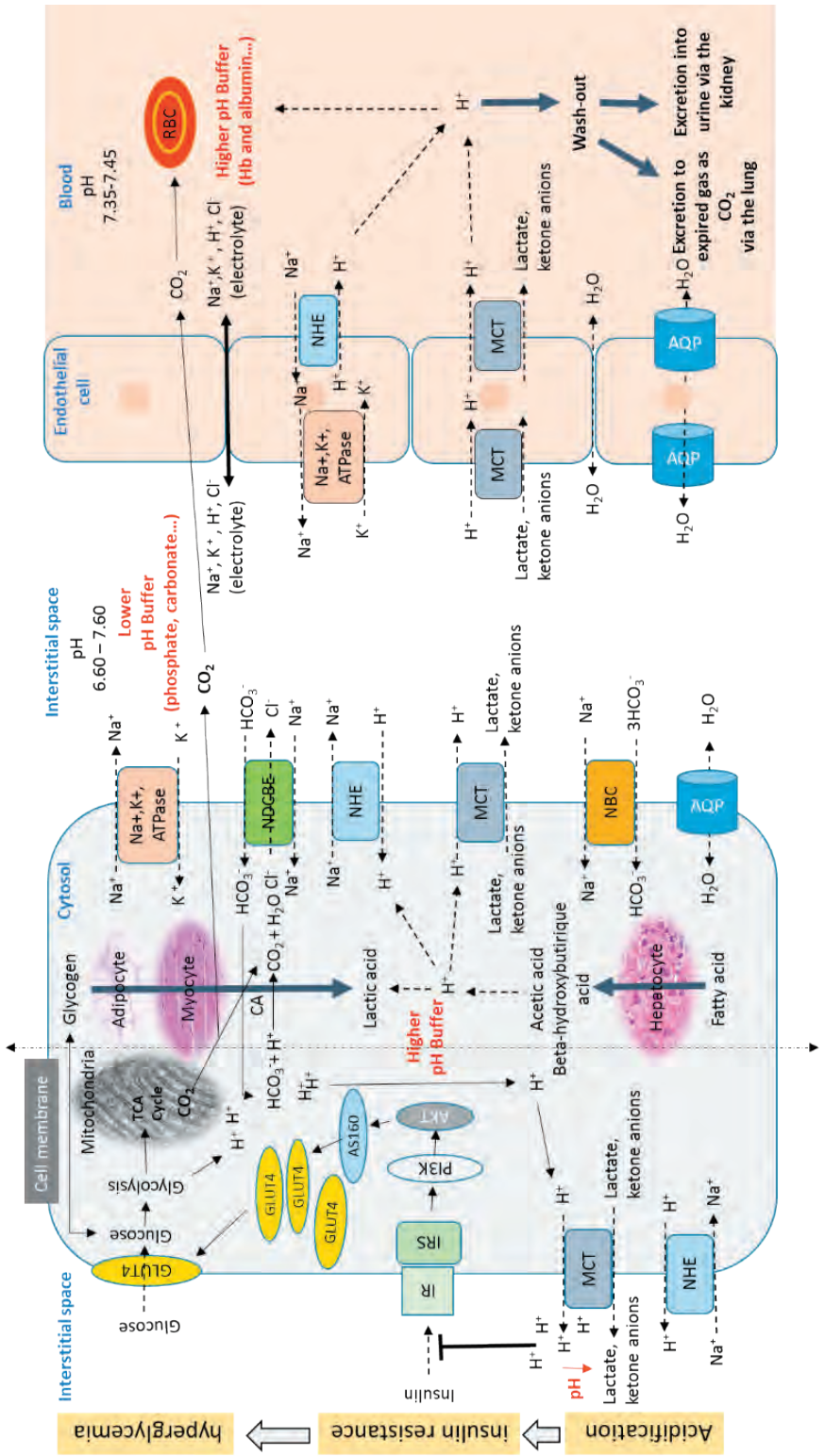


Figure 1: pH homeostasis of the most cells involved in the physiological and pathological glucose metabolism. Living cells are actively releasing proton from the metabolism of organic acid molecules that decide the pH of body fluid. Throughout metabolic tissues such as the skeletal muscles and adipose tissues, the glycolytic metabolism of glucose and glycogen under anaerobic conditions produces lactic acid (lactate$^-$/H$^+$) while the excess of fatty acid metabolism of hepatocytes promotes the production of ketone bodies, beta-hydroxybutyric and acetoacetate acids. Cells procures many ways to remove excess proton production to maintain pH homeostasis of cytosol, interstitial liquid and blood system. These PH buffering mechanisms, are assisted by many co-transporters and proteins exchangers, Na+-driven Cl$^-$/HCO_{3-} exchanger (NDCBE) and Na+-HCO_{3-} cotransporter (NBC) participate in uptake of HCO_{3-} from interstitial space into intracellular. Monocarboxylate Transporter (MCT) and Na$^+$/H$^+$ Exchanger (NHE) remove proton from the cytosol to the extracellular space supported indirectly by Na$^+$/K$^+$ ATPase pomp. In fact, protons were transported to circulation by endothelial cell transport systems. Though, in comparison to intracellular fluid and blood (high pH buffer), the interstitial fluid may quickly become acidic due to its low pH buffering capacity such as proteins. However, in skeletal muscle cells glucose and glycogen are the main energy substrates. Excessive glucose / glycogen consumption and aerobic metabolism deficiency induces lactic acid production via the glycolysis pathway leading to interstitial pH depletion. This latter, affect the binding affinity of insulin to its receptor and the glucose uptake via Glucose Transporter 4 (GLUT4). Weak interstitial fluid pH therefore induces insulin resistance and hyperglycemia by reducing insulin sensitivity to it receptor in skeletal muscle cells. IR, insulin receptor; IRS, insulin receptor substrate; PI3 K, phosphoinositide-3-kinase; AS160, Act substrate, 160 kDa; CA, carbonic anhydrase; AQP, aquaporin.

have been associated with a substantially lower risk of insulin resistance and type 2 diabetes and improved glycemic control in either normal or insulin-resistant individuals [33] (Fig. 1).

Nonetheless, vegetables and fruits are more than an excellent alkaline diet. Indeed, legumes also supply organisms with a slow-release carbohydrate and are rich in soluble fiber and factors known to improve glycemic control and overweight such as polyphenols. In related references it was deduced that a decrease in serum HbA1C levels in T2DM is correlated strongly with decrease in body weight. In contrast, poorly planned vegetarian diets can be deficient in vitamin B12, calcium, vitamin D, zinc, iron, and long-chain omega-3 fatty acids. Vegetarians need to incorporate into their diet foods that provide adequate levels of these vitamins, minerals, and omega-3 fatty acids [33]. Additionally, concerning glycemic responses to different carbohydrate foods, there exists a relationship between the rate of digestion of starchy foods in vitro and the glycemic response to them in vivo. These differences are linked to many factors that influence the rate of digestion, including the nature of the starch, the food form, the content and type of dietary fiber, and the presence of the so-called anti-nutrients [26]. In consequence, because these factors are not adequately listed in food tables, it is not possible to predict exactly the glycemic effect or acid-base balance of a food based only on its chemical composition.

Acid load and endocrine disorders

In addition to insulin resistance, many other disturbances in endocrine metabolism have been described. In fact, metabolic acidosis is observed to increase glucocorticoid secretion and decreases the accumulation of cortisol in both plasma and urine [34, 35]. Excess cortisol in metabolic acidosis can lead to insulin resistance, proteolysis [31] and increase urinary ammonium excretion resulting from protein degradation pathway [36]. Further, a contemporary acidogenic diet is also linked to excess cortisol but bicarbonate administration is associated with a significant reduction of plasma cortisol level and free cortisol urinary excretion [31]. Likewise, diabetes acidosis is marked to decrease circulating rate of IGF-1 due to peripheral growth hormone insensitivity, but at the same time it's shown to increase plasma leptin level. This latter hormone is also known for its stimulating effect on satiety, increasing uremic anorexia syndrome and energy expenditure, and finally on reduction of cardiovascular risk [23]. Parathyroid hormone (PTH) is known to contribute to acid base balance by increasing bicarbonate excretion via inhibiting proximal bicarbonate reabsorption. This effect is demonstrated by the experiment of Arruda et al. [37]. This experiment was performed on 105 rats, which demonstrate that both PTH forms, exogenous or endogenous, play a central role in buffering of acid load via its influence on carbonic anhydrase [37]. The metabolism of different foods releases or consumes H^+ ions [23].

Acidosis and oral antidiabetic treatment responses

The increased glycemic level is not the only important sign in the pathogenesis of T2D, but is the result of insulin resistance [5]. Indeed, all oral antidiabetic drugs (OADs) are primarily designed to reduce blood glucose levels by blocking glucose production, glucose reuptake or stimulating insulin release [38], but not to essentially improve insulin resistance. For example, biguanide blocks gluconeogenesis from lactate mainly in the liver; sulfonylurea stimulates pancreatic β cells to release insulin; glucosidase inhibitors reduces the intestine breakdown of carbohydrate by inhibiting glucosidases; thiazolidine stimulates adiponectin release from adipocytes leading to glucose uptake by acting peroxisome proliferator-activated receptor (PPAR); dipeptidyl-peptidase IV (DPP-4) inhibitors preserve a high level of insulin release from pancreatic β cells by blocking break-down of incretin in the intestine. Furthermore, sodium-glucose cotransporter 2 (SGLT2) inhibitors reduce kidney's epithelium reuptake of glucose [5] independently to insulin secretion or resistance [38].

On the other side, a tremendous variability in the effect of antidiabetic drugs was observed between diabetic subjects [39]. This

https://doi.org/10.46389/rjd-2020-1060
© 2020 The Authors

heterogeneity of treatment effects refers to the differential response to the same treatment by different patients. More specifically, this definition includes different responses of patients with different characteristics. In the literature, several theories have been proposed to explain those characteristics which can include severity of the disease under study (severe versus milder forms of the same disease); socio-demographic characteristics, such as age, sex, and race/ethnicity; genetic characteristics; and health-related behaviors, such as adherence to treatment, alcohol consumption, and use of complementary or alternative medicine [40]. Additionally, biological and non-genetic factors such as intestinal absorption, liver and kidney function or pharmacokinetic drug interactions may occur [39].

Then, significant differences between patients in terms of glucose-lowering response to OADs, tolerability and drugs incidence of adverse events is also indicated [3, 41]. To highlight this latter, metformin-associated lactic acidosis is a rare but significant adverse event and it is critical to unraveling the problem. First, this potential event continues to influence treatment strategies for T2DM, especially in many high risk patients with kidney failure, those with metformin contraindications and elderly patients [42]. Lactic acidosis is the most common cause of metabolic acidosis. It is marked by an increase in the anion gap (the concentration of sodium in the blood minus the chloride and bicarbonate concentrations) [43]. As to biguanides, they have a marked effect on glucose/lactate metabolism. Its antidiabetic properties result from the blockade of gluconeogenic precursors, such as alanine and lactate, to pyruvate. This effect is missed in other classes of antidiabetic medicines. Furthermore, all biguanides are strong bases which are fully protonated at physiological pH. Their two-dimensional structures suggest close similarities between members of this class. However, the slight differences, lead to profound variances in the behavior of these molecules in solution and also in terms of their pharmacokinetics and metabolism [43].

Faerch et al. [44] demonstrate that drugs used to treat T2DM have been a marked interaction with lifestyle and patients environment; for example, the hypoglycemic drugs such as sulfonylureas, thiazolidinediones, biguanides, and as well as lipid-lowering statins, have each been shown to interact with physical activity to influence insulin secretion, insulin sensitivity, or glycemic control [44]. The influence of each factor (genetic and epigenetic) on the development of the disease is extremely important. In fact, we cannot edit the structure of our genes, but we can influence the outcomes of their expression, e.g., through epigenetic modulation of transcription (development or prevention of the disease, drug metabolism) and through our lifestyle, in order to prevent or delay the onset of abnormalities [41].

Lower insulin sensitivity is observed in lower levels of serum bicarbonate and higher levels of anions resulting from metabolic acidosis. In fact, Mandel and colleagues [45], showed in the Nurses' Health Study a relationship between rise of plasma bicarbonate levels and reduced risk of incident T2DM among women who developed the disease after 10 years of monitoring [45]. The most interesting approach to this issue has been proposed in the work of Laboux and Azar [23], which reveal that the normalization of the plasma bicarbonate level is associated with a reduction in insulin dose and OADs posology of patients with T2DM [23]. Craig [33] has also suggested, that vegan diet characterized by low fats, poor glycemic load, and rich on fiber is observed to improved considerably glycemic control of T2DM. This funding is associated with a reduction about 43% of diabetes medication after only five months of diet [33] (Fig.2). Very few publications are available in the literature that discuss the improvement of OADs responses by an alkaline diet in acidosis situations. Further experimental confirmation of this theory on the issue would be of interest.

Conclusion

Quite recently, considerable attention has been yielded to the impact of food intake and its acid load in different pathological situation. According to this review, we conclude that the fall of interstitial fluid pH (weak pH buffering capacity) observed in T2DM leads to insulin resistance

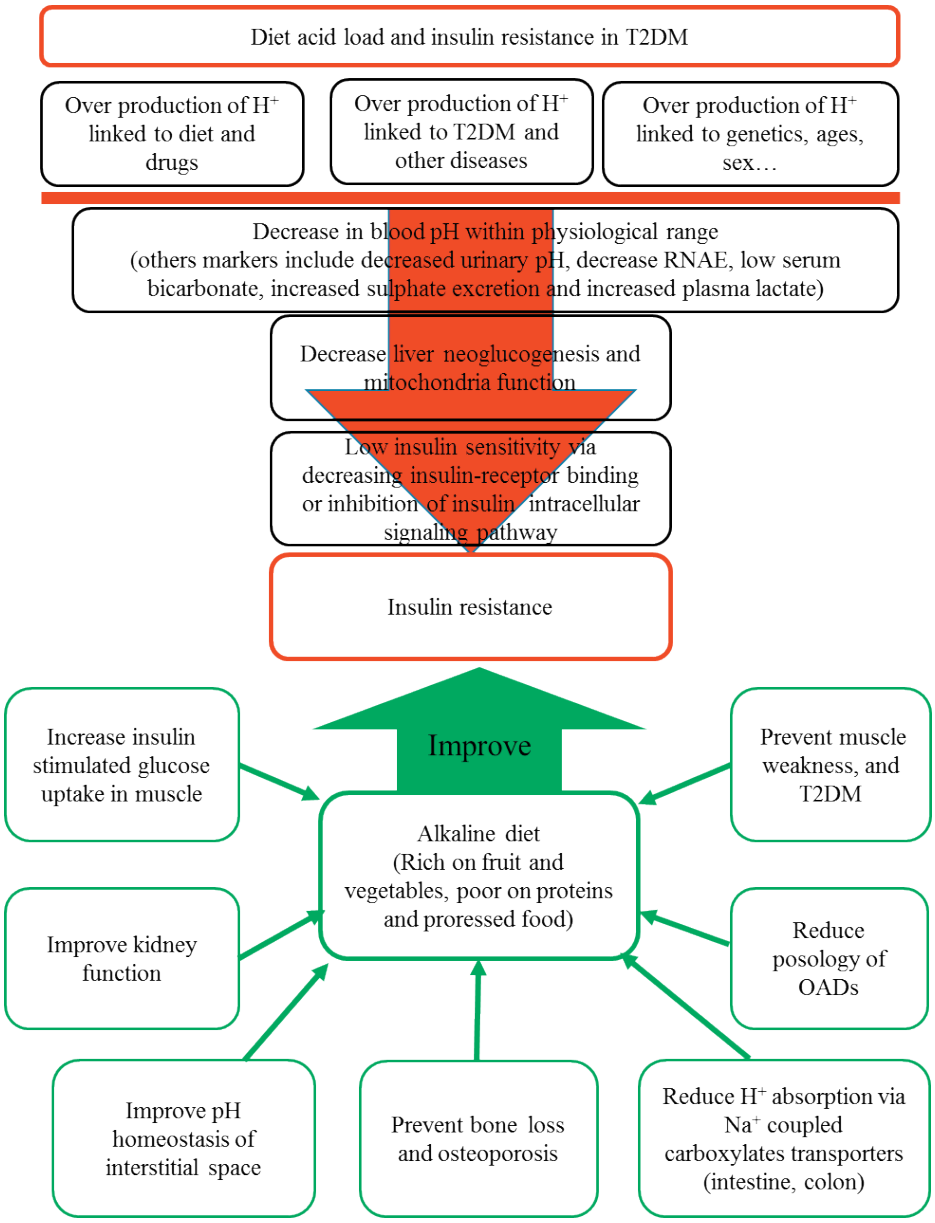

Diet acid load and insulin resistance in T2DM
Over production of H+ linked to diet and drugs
Over production of H+ linked to T2DM and other diseases
Over production of H+ linked to genetics, ages, sex…
Decrease in blood pH within physiological range (others markers include decreased urinary pH, decrease RNAE, low serum bicarbonate, increased sulphate excretion and increased plasma lactate)
Decrease liver neoglucogenesis and mitochondria function
Low insulin sensitivity via decreasing insulin-receptor binding or inhibition of insulin intracellular signaling pathway
Insulin resistance
Improve
Increase insulin stimulated glucose uptake in muscle
Prevent muscle weakness, and T2DM
Alkaline diet (Rich on fruit and vegetables, poor on proteins and proressed food)
Improve kidney function
Reduce posology of OADs
Improve pH homeostasis of interstitial space
Prevent bone loss and osteoporosis
Reduce H+ absorption via Na+ coupled carboxylates transporters (intestine, colon)

Figure 2: Summarizes dietary alkaline-acid load effects on body metabolism and insulin resistance in humans.

via reduced insulin-receptor binding affinity or via the reduction of insulin affinity with its receptor or via the impairment of intracellular insulin signaling pathway and mitochondria metabolism. Then, considering the evidence in the literature, we can believe that alkaline diets (fruits and vegetables) notably rich in vegetal proteins, soluble fiber, and low glycemic index associated with poor processed food can reduce the level of acidic load in the body and ameliorate the response of diabetic's patients to OADs. Thus, it could improve hyperglycemia and metabolic complications resulting mainly from disturbance of interstitial space pH buffering. Consequently, evaluation of acid diet load and the use of diet replacement feature (replacement of acidic food with another less acidic, referring to the table above of calculating PRAL) can be an important approach for future interventions in populations with a high risk for T2DM, especially for elderly persons. It may also contribute to the control of the body acidosis and reducing posology and side effects of OADs in this group. The next stage of our research will be experimental to confirm this theory concept.

https://doi.org/10.46389/rjd-2020-1060
© 2020 The Authors

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

There is no financial support for this work

References

1. Akter S, Eguchi M, Kuwahara K, Kochi T et al. High dietary acid load is associated with insulin resistance: The Furukawa Nutrition and Health Study. *Clin Nutr*. 35(2):453–459, 2016.
2. Forouhi NG, Misra A, Mohan V, Taylor R et al. Dietary and nutritional approaches for prevention and management of type 2 diabetes. *BMJ*. 361:k2234, 2018.
3. Emami-Riedmaier A, Schaeffeler E, Nies A, Mörike K et al. Stratified medicine for the use of antidiabetic medication in treatment of type II diabetes and cancer: where do we go from here? *J Intern Med*. 277(2):235–247, 2015.
4. Shi G-J, Li Y, Cao Q-H, Wu H-X et al. In vitro and in vivo evidence that quercetin protects against diabetes and its complications: A systematic review of the literature. *Biomed Pharmacother*. 109:1085–1099, 2019.
5. Marunaka Y. Roles of interstitial fluid pH in diabetes mellitus: Glycolysis and mitochondrial function. *World J Diabetes*. 6(1):125, 2015.
6. Marunaka Y. The proposal of molecular mechanisms of weak organic acids intake-induced improvement of insulin resistance in diabetes mellitus via elevation of interstitial fluid pH. *Int J Mol Sci*. 19(10):32–44, 2018.
7. Osuna-Padilla I, Leal-Escobar G, Garza-García C, Rodríguez-Castellanos F. Dietary Acid Load: Mechanisms and evidence of its health repercussions. *Nefrología*. 39(4):343–354, 2019.
8. Gillies RJ, Pilot C, Marunaka Y, Fais S. Targeting acidity in cancer and diabetes. *Biochim Biophys Acta Rev Cancer*. 1871(2):273–280, 2019.
9. Aoi W, Zou X, Xiao JB, Marunaka Y. Body Fluid pH Balance in Metabolic Health and Possible Benefits of Dietary Alkaline Foods. *eFood*. 1(1):12–23, 2019.
10. Puddu A, Sanguineti R, Montecucco F, Viviani GL. Evidence for the gut microbiota short-chain fatty acids as key pathophysiological molecules improving diabetes. *Mediators Inflamm*. 2014:1–9, 2014.
11. Williams RS, Kozan P, Samocha-Bonet D. The role of dietary acid load and mild metabolic acidosis in insulin resistance in humans. *Biochimie* 124:171–177, 2016.
12. Newman JC, Verdin E. β-Hydroxybutyrate: a signaling metabolite. *Annu Rev Nutr* 37:51–76, 2017.
13. Sperelakis N. Intracellular pH regulation. In: *Cell physiology source book: essentials of membrane biophysics*. Press Academic (Ed). Canada, Elsevier, pp 303–321, 2012.
14. McNamara J, Worthley L. Acid-base balance: part I. Physiology. *Crit Care Resusc*. 3:181–187, 2001.
15. Busa W, Nuccitelli R. Metabolic regulation via intracellular pH. *Am J Physiol Regul Integr Comp Physiol*. 246(4):R409–R438, 1984.
16. Schwalfenberg GK. The alkaline diet: is there evidence that an alkaline pH diet benefits health? *J Environ Public Health*. 2012:1–7, 2012.
17. Barzel US, Massey LK. Excess dietary protein can adversely affect bone. *J Nutr*. 128(6):1051–1053, 1998.
18. El-Hattab AW, Emrick LT, Hsu JW, Chanprasert S et al. Glucose metabolism derangements in adults with the MELAS m. 3243A> G mutation. *Mitochondrion*. 18:63–69, 2014.
19. Fríguls B, Joya X, García-Algar O, Pallás C et al. A comprehensive review of assay methods to determine drugs in breast milk and the safety of breastfeeding when taking drugs. *Anal Bioanal Chem*. 397(3):1157–1179, 2010.
20. Demaurex N. pH Homeostasis of cellular organelles. *Physiology* 17(1):1–5, 2002.
21. Banerjee T, Crews DC, Wesson DE, Tilea A et al. Dietary acid load and chronic kidney disease among adults in the United States. *BMC Nephrol*. 15(1):137, 2014.
22. Angéloco LRN, de Souza GCA, Romão EA, Chiarello PG. Alkaline diet and metabolic acidosis: practical approaches to the nutritional management of chronic kidney disease. *J Ren Nutr*. 28(3):215–220, 2018.
23. Laboux T, Azar R. Dietary control of metabolic acidosis in chronic kidney disease. *Nephrol Ther*. 15(7):491–497, 2019.
24. Remer T, Dimitriou T, Manz F. Dietary potential renal acid load and renal net acid excretion in healthy, free-living children and adolescents. *Am J Clin Nutr*. 77(5):1255–1260, 2003.
25. Frassetto LA, Todd KM, Morris Jr RC, Sebastian A. Estimation of net endogenous noncarbonic acid production in humans from diet potassium and protein contents. *Am J Clin Nutr*. 68(3):576–583, 1998.
26. Chauveau P, Lasseur C, Nodimar C, Prezelin-Reydit M et al. Dietary acid load: A novel target for the nephrologist? *Nephrol Ther*. 14(4):240–246, 2018.
27. Passey C. Reducing the dietary acid load: How a more alkaline diet benefits patients with chronic kidney disease. *J Ren Nutr* 27(3):151–160, 2017.
28. Remer T, Manz F. Potential renal acid load of foods and its influence on urine pH. *J Am Diet Assoc*. 95(7):791–797, 1995.
29. Menezes CJ, Worcester EM, Coe FL, Asplin J et al. Mechanisms for falling urine pH with age in stone formers. *Am J Physiol Renal Physiol*. 317(7):65–72, 2019.
30. Frassetto L, Sebastian A. Age and systemic acid-base equilibrium: analysis of published data. *J Gerontol A Biol Sci Med Sci*. 51(1):91–99, 1996.
31. Adeva MM, Souto G. Diet-induced metabolic acidosis. *Clin Nutr*. 30(4):416–421, 2011.
32. Williams RS, Heilbronn LK, Chen DL, Coster AC et al. Dietary acid load, metabolic acidosis and insulin resistance–Lessons from cross-sectional and overfeeding studies in humans. *Clin Nutr*. 35(5):1084–1090, 2016.
33. Craig WJ. Nutrition concerns and health effects of vegetarian diets. *Nutr Clin Pract*. 25(6):613–620, 2010.
34. Maurer M, Riesen W, Muser J, Hulter HN et al. Neutralization of Western diet inhibits bone resorption independently of K intake and reduces cortisol secretion in humans. *Am J Physiol Renal Physiol*. 284(1):32–40, 2003.

© 2020 The Authors

35. Ballmer PE, Imoberdorf R. Influence of acidosis on protein metabolism. *Nutr Clin Pract*. 11(5):462–470, 1995.
36. Defronzo RA, Beckles A. Glucose intolerance following chronic metabolic acidosis in man. *Am J Physiol Endocrinol Metab*. 236(4):E328, 1979.
37. Arruda J, Alla V, Rubinstein H, Cruz-Soto M et al. Parathyroid hormone and extrarenal acid buffering. *Am J Physiol Renal Physiol*. 239(6):533–538, 1980.
38. Pillon F, Tan K, Jouty P, Frullani YJAp. Le traitement médicamenteux du diabète de type 2. 53(541):23–28, 2014.
39. Becker ML, Pearson ER, Tkáč I. Pharmacogenetics of oral antidiabetic drugs. *Int J Endocrinol*. 2013:1–10, 2013.
40. Greenfield S, Kravitz R, Duan N, Kaplan SH. Heterogeneity of treatment effects: implications for guidelines, payment, and quality assessment. *Am J Med*. 120(4):3–9, 2007.
41. Tremblay J, Hamet P. Environmental and genetic contributions to diabetes. *Metabolism*. 100:153952, 2019.
42. May M, Schindler C. Clinically and pharmacologically relevant interactions of antidiabetic drugs. *Ther Adv Endocrinol Metab*. 7(2):69–83, 2016.
43. Lalau JD. Lactic acidosis induced by metformin. *Drug Saf*. 33(9):727–740, 2010.
44. Faerch K, Hulman A, PJ Solomon T. Heterogeneity of pre-diabetes and type 2 diabetes: implications for prediction, prevention and treatment responsiveness. *Curr Diabetes Rev*. 12(1):30–41, 2016.
45. Mandel EI, Curhan GC, Hu FB, Taylor EN. Plasma bicarbonate and risk of type 2 diabetes mellitus. *CMAJ*. 184(13):719–725, 2012.

 https://doi.org/10.46389/rjd-2020-1060 © 2020 The Authors

Societatea Română de Diabet, Nutriție și Boli Metabolice

Organizator științific

SOCIETATEA ROMÂNĂ DE DIABET, NUTRIȚIE, BOLI METABOLICE
1957

47

AL 47-lea CONGRES NAȚIONAL al Societății Române de Diabet, Nutriție și Boli Metabolice

19-22 mai 2021

Producție online &
Management eveniment

Sănătatea PRESS GROUP
Expertul tău în comunicare în domeniul sănătății

CONGRES DE TIP WEBINAR CU PARTICIPARE INTERNAȚIONALĂ
Exclusiv ONLINE, pe www.Sanatatea.ONLINE/learning

Pompa MiniMed™ 780G

SISTEMUL MINIMED™ 780G AUTOMATIZAT PENTRU A AJUTA MAI MULȚI PACIENȚI SĂ ÎȘI ATINGĂ ȚINTELE GLICEMICE CU MAI PUȚIN EFORT[1-4]

Medtronic

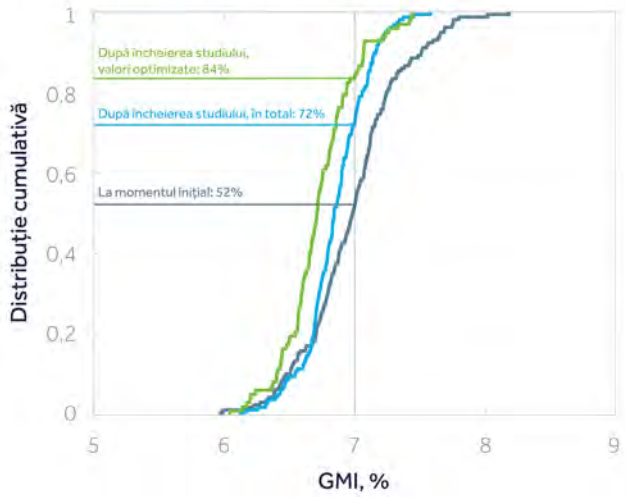
REFERINȚE

* Consultați Ghidul Utilizatorului Sistemului - componentul SmartGuard. Este necesară interacțiunea utilizatorului

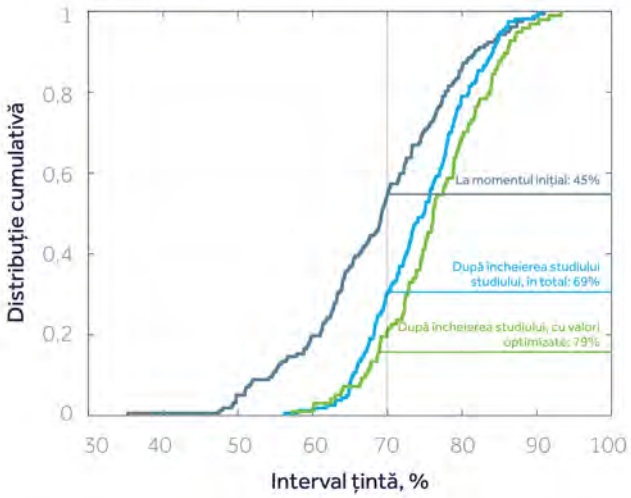
1. Carlson, AL. et al. Poster at the 80th International Conference of the American Diabetes Association, June 12-16. 2020, Chicago/Virtual
2. De Bock M. et al. Poster at the 80th International Conference of the American Diabetes Association, June 12-16. 2020, Chicago/Virtual
3. Battelino T, et al. Diabetes Care 2019;42(8): 1593-1603
4. ADA Guidelines https://www.diabetes.org/a1c
5. Collyns O. et al. Poster at the 80th International Conference of the American Diabetes Association, June 12-16. 2020, Chicago/Virtual

SISTEMUL DE POMPĂ DE INSULINĂ CU RATĂ BAZALĂ AUTO-REGLABILĂ ȘI DOZAJ PENTRU AUTO-CORECȚIE*

84% dintre pacienți și-au atins ținta GMI (indicator glucoză) după perioada de studiu cu valori optimizate în comparație cu **52%** la momentul inițial

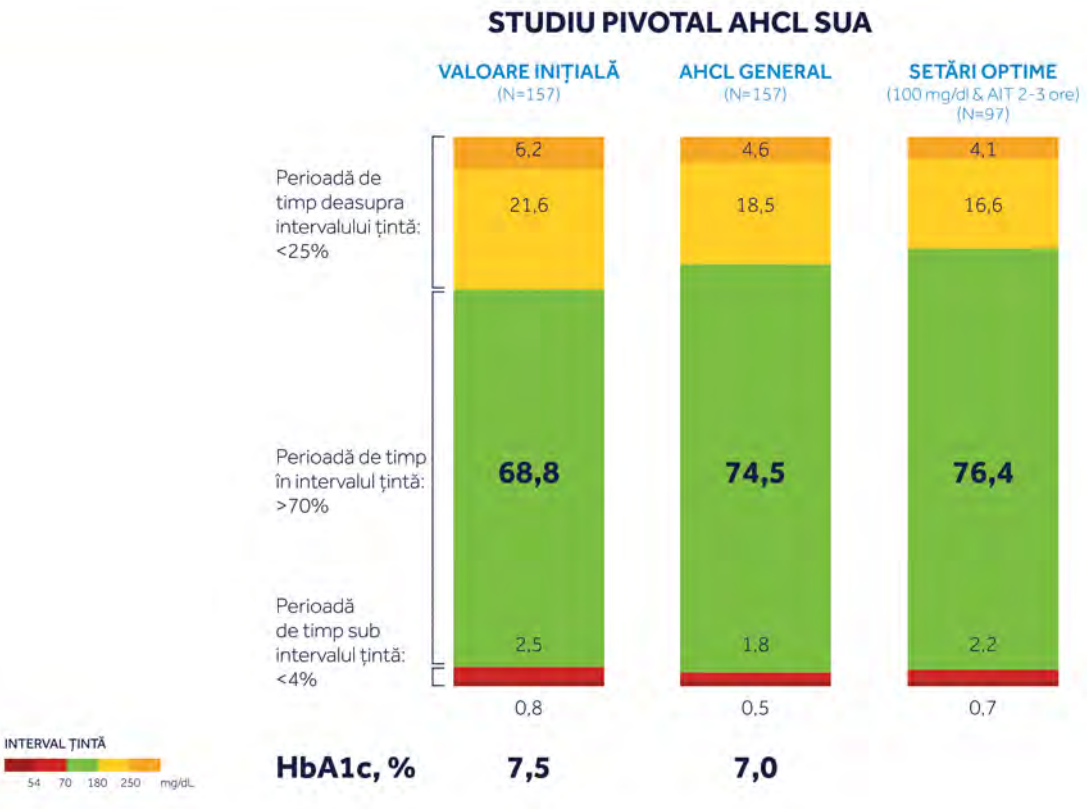


79% dintre pacienți au atins intervalul țintă după încheierea studiului cu valori optimizate în comparație cu **45%** la momentul inițial



La momentul inițial | După încheierea studiului, în total | După încheierea studiului, la ținta de 100 mg/dl + AIT 2-3 ore

REZULTATELE SUPERIOARE AJUTĂ LA ATINGEREA OBIECTIVELOR PRIVIND INTERVALUL ȚINTĂ ȘI HbA1c[1,3,4,5]



baqsimi®

(glucagon) pulbere nazală

PREGĂTIT ORICÂND PENTRU A SALVA

SINGURUL GLUCAGON CU ADMINISTRARE NAZALĂ

- BAQSIMI® este indicat pentru tratamentul hipoglicemiei severe la adulți, adolescenți și copii începând cu vârsta de 4 ani și peste, cu diabet zaharat.[1]

- BAQSIMI® este un tratament inovator pentru hipoglicemia severă, ce poate fi administrat cu succes, ușor și rapid în caz de urgență, inclusiv de către persoane fără pregătire medicală (membrii familiei, rude, prieteni, colegi)[1,2]

*Despre **BAQSIMI®**[1-8]*

- Baqsimi 3mg (glucagon, pulbere nazală) în flacon cu doză unică, compact, portabil[1-7]
- Gata de utilizare, nu necesită preparare sau reconstituire[1-7]
- Absorbție pasivă prin mucoasa nazală; se administrează într-o singură nară[1-7]
- Poate fi utilizat chiar și în prezența congestiei nazale[8]
- Poate fi păstrat în ambalajul original la o temperatură nu mai mare de 30°C[1]

S.C. ELI LILLY ROMANIA S.R.L.,
Strada Menuetului, nr. 12, Bucharest Business Park,
Corp D, Etaj 2, 013713, Sector 1, București,
Tel: 021-402.30.00, Fax: 021-402.30.01.

Lilly

baqsimi®

(glucagon) pulbere nazală

Administrare ***BAQSIMI***[9]

Pregătirea pentru administrarea dozei:

- Îndepărtați folia protectoare trăgând de banda roșie
- Deschideți capacul și scoateți flaconul cu doza unică din tub

Administrarea dozei:

ȚINEȚI flaconul cu doza unică ca în imagine. **Nu apăsați pistonul înainte de introducerea în nară.**

INTRODUCEȚI vârful flaconului într-una din nări

APĂSAȚI pistonul complet **până când linia verde nu mai este vizibilă.**

Referințe: **1.** Baqsimi® – RCP, decembrie 2019; **2.** Settles JA et al. Endocr Pract. 2020 Apr;26(4):407-415; Sherr JL, et al. Diabetes Care 2016;39(4):555-62; **4.** Rickels MR, et al. Diabetes Care. 2016:39(2):264-70; **5.** Seaquist ER, et al. Diabetes Obes Metab. 2018;20(5):1316-20; **6.** Deeb LC, et al. Pediatr Diabetes. 2018:19(5):1007-13; **7.** Yale JF, et al. Diabetes Technol Ther. 2017;19(7):423-32; **8.** Guzman CB, et al. Diabetes Obes. Metab. 2018;20:646-53.; **9.** Prospect de administrare Baqsimi, varianta aprobata ANMDMR, aprilie 2020

Material destinat profesioniștilor din domeniul sănătății.

PP-GN-RO-0017

Pentru mai multe detalii despre BAQSIMI®, consultați Rezumatul Caractristicilor Produsului scanândcodul QR alăturat.

Rolul magneziului în obezitate, sindromul metabolic și diabetul zaharat tip 2

Dr. Cătălin Colț
Medic rezident Medicină Sportivă, Institutul Național de Medicină Sportivă, București

Magneziul este al 2-lea cel mai abundent cation intracelular și al 4-lea cel mai abundent cation din corpul uman. Din întreaga cantitate de magneziu din organism, cea mai mare parte (60%) se găsește în oase și țesuturile moi (40%). Doar un mic procent (sub 1%) este prezent la nivelul sângelui. Este cofactor pentru sute se reacții enzimatice, exemplul cel mai cunoscut fiind complexul Mg-ATP, în care ionul de Mg2+ se leagă de adenozin trifosfat (ATP) și formează principala sursă de energie celulară. În prezent, se estimează că sunt peste 600 de enzime pentru care magneziul servește ca și cofactor și peste 200 pentru care magneziul acționează ca și activator. Multe dintre enzimele care au magneziul ca și cofactor sunt vitale pentru viață. Printre enzimele dependente de magneziu se numără: Na+/K+ ATP-aza, creatinkinaza, hexokinaza, proteinkinaza și adenilat ciclaza. Magneziul este implicat, printre altele, în metabolismul energetic și cel al carbohidraților. Mai mult, magneziul este esențial pentru metabolismul intermediar din sinteza macromoleculelor. Magneziul ajută la menținerea funcției normale a nervilor și mușchilor dar și a ritmului cardiac, tonusului vasomotor, tensiunii arteriale, nivelului glicemiei, metabolismului osos și a imunității.

Obezitatea, diabetul zaharat de tip 2 și sindromul metabolic sunt trei entități strâns legate între ele printr-o serie de mecanisme fiziopatologice care au la bază inflamația sistemică „de grad redus". Depleția de magneziu poate produce inflamație fie direct, fie indirect prin modificarea microbiotei intestinale. Deficitul de magneziu apare frecvent la persoanele obeze și are o prevalență crescută la persoanele cu sindrom metabolic și diabet zaharat tip 2. Mai mult, deficitul de magneziu crește riscul de apariție a diabetului zaharat tip 2.

Obezitatea și comorbiditățile sale reprezintă o problemă importantă de sănătate peste tot în lume. Ea este rezultatul unei diete nesănătoase, cu conținut caloric ridicat dar săracă în nutrienți. Ca o consecință firească, persoanele obeze prezintă adesea deficit de magneziu. Studii efectuate în S.U.A. au arătat că deficitul de Mg este mai des întâlnit la persoanele cu indicele de masă corporală (IMC) în intervalul corespunzător pentru obezitate iar un studiu din Franța a relevat că aportul de magneziu este afectat la 35% dintre indivizii cu IMC > 35. Studiul CARDIA, care s-a întins de-a lungul a 30 de ani și a înrolat mai mult de 5.000 de persoane, a arătat că aportul de magneziu este invers proporțional cu incidența obezității și nivelul proteinei C reactive (CRP). Alte studii au raportat o asociere inversă între aportul de magneziu și IMC, respectiv circumferința taliei, iar pe modele de obezitate indusă prin dietă la animale, suplimentarea cu magneziu a prevenit acumularea de țesut adipos.

În plus, persoanele obeze, fie că au sau nu asociat diabet zaharat de tip 2, au adesea și deficit de vitamina D, iar magneziul este esențial pentru sinteza și activarea acesteia. Din cauza rolului său în sistemul renină-angiotensină și a proprietăților sale imunomodulatoare, deficitul de vitamină D reprezintă un potențial factor de risc pentru tulburări cardiometabolice printre care: rezistența la insulină, sindromul metabolic și boli cardiovasculare. De asemenea, deficitul cronic de magneziu concomitent cu deficiența vitaminei D predispune indivizii obezi, non-diabetici, la un risc crescut de boli cardiometabolice. Pe de altă parte, menținerea unui nivel optim de magneziu îmbunătățește efectele benefice ale vitaminei D asupra factorilor de risc cardiometabolici.

Sindromul metabolic, la fel ca și obezitatea, se caracterizează prin acumularea de grăsime în organism. Dar, spre deosebire de obezitate, sindromul metabolic implică un proces inflamator sistemic și asocierea a cel puțin trei din următoarele entități:

- obezitate de tip central – definită prin creșterea circumferinței abdominale peste 102 cm la bărbați și peste 88 cm la femei
- toleranță alterată la glucoză – glicemia a jeun ≥ 110 mg/dl
- creșterea trigliceridelor – peste 150 mg/dl
- scăderea HDL-colesterolului - sub 40 mg/dl la bărbați și sub 50 mg/dl la femei
- creșterea valorilor tensiunii arteriale – peste 130/85 mmHg

Numeroase studii au relevat că există o relație directă între aportul de magneziu și riscul de apariție a sindromului metabolic, independent de alți factori de risc: vârsta, sexul, IMC, fumatul, consumul de alcool, etc. Dibaba și colab. au arătat într-o meta-analiză recentă că aportul de magneziu este invers asociat cu prevalența sindromului metabolic. Într-un studiu desfășurat în China, pe 11.000 de femei cu vârsta de peste 45 de ani, s-a observat că aportul crescut de magneziu a fost asociat cu o scădere a proteinei C reactive, a inflamației sistemice și a riscului de apariție a sindromului metabolic, în timp ce un interesant studiu din Serbia a găsit o corelație între consumul de apă potabilă săracă în magneziu și prevalența crescută a hipertensiunii arteriale și a sindromului metabolic.

Magneziul este un blocant natural de calciu, date experimentale recente sugerând că un raport calciu/magneziu crescut și dezechilibrat în favoarea calciului, indus de o dietă bogată în calciu și săracă în magneziu poate duce la hipertensiune arterială, rezistență la insulină și sindrom metabolic.

Diabetul zaharat este o enormă și globală problemă de sănătate publică. La nivelul anului 2019 existau nu mai puțin de 463 de milioane de persoane cu diabet zaharat (9,3% din populația globului) iar prognoza pentru anul 2045 estimează că peste 700 de milioane de persoane vor avea această boală (10,9% din populația lumii). Tot în anul 2019, diabetul zaharat și complicațiile acestuia au cauzat 4,2 milioane de decese iar cheltuielile anuale de sănătate legate de această afecțiune s-au situat la nivelul a 760 de miliarde de dolari.

Diabetul zaharat de tip 2 este adesea asociat cu alterarea homeostaziei magneziului, în timp ce numeroase studii au arătat o prevalență ridicată a hipomagneziemiei la persoanele care suferă de această afecțiune. Deficitul de magneziu la persoanele cu diabet zaharat tip 2 este cauzat în principal de un aport scăzut și de pierderi urinare crescute de magneziu, probabil cauzate de afectarea funcției renale. Mai mult, este demonstrat că hipomagneziemia este asociată cu progresia diabetului zaharat și apariția complicațiilor. Relația dintre magneziu și diabetul zaharat poate fi explicată prin influența pe care magneziul o are asupra metabolismului glucozei și a sensibilității la insulină. Deficitul de magneziu este de asemenea asociat cu dezvoltarea rezistenței la insulină, fenomen descris inclusiv în cazul copiilor și adolescenților.

Luând în considerare prevalența foarte mare a diabetului zaharat de tip 2, a sindromului metabolic și a obezității, corectarea obiceiurilor alimentare nesănătoase și suplimentarea dietei zilnice cu magneziu poate preveni apariția acestor afecțiuni sau poate încetini progresia lor spre complicații serioase.

Referințe

1. Piuri, G.; Zocchi, M.; Della Porta, M.; Ficara, V.; Manoni, M.; Zuccotti, G.V.; Pinotti, L.; Maier, J.A.; Cazzola, R. Magnesium in Obesity, Metabolic Syndrome, and Type 2 Diabetes. Nutrients 2021, 13, 320.
2. Dominguez, L.J.; Veronese, N.; Barbagallo, M. Magnesium and Hypertension in Old Age. Nutrients 2021, 13, 139.
3. Song, Y.; Ridker, P.M.; Manson, J.A.E.; Cook, N.R.; Buring, J.E.; Liu, S. Magnesium intake, C-reactive protein, and the prevalence of metabolic syndrome in middle-aged and older U.S. women. Diabetes Care 2005, 28, 1438–1444.
4. IDF Diabetes Atlas, 9th edition 2019; disponibil la https://www.idf.org/e-library/epidemiology-research/diabetes-atlas

CÂND EȘTI STRESAT

DEVII STRESANT

Magnerot® combate stresul, oboseala cronică și epuizarea. **Magnerot®** conține **orotat de magneziu**, care se absoarbe rapid în organism și te ajută să depășești perioadele de suprasolicitare.

www.magnerot.ro

Magnerot 500 mg comprimate este un medicament ce conține 500 mg orotat de magneziu dihidrat (echivalent a 32,8 mg magneziu) indicat în profilaxia infarctului miocardic şi necrozei miocardice, în tratamentul anginei pectorale, dacă aceste afecţiuni sunt determinate de carenţa de magneziu, arterioscleroză, stenocardie, arterită şi arteriolită, tulburări ale metabolismului lipidic, crampe musculare. Se administrează timp de o săptămână câte 2 comprimate (1g orotat de magneziu) de 3 ori pe zi, pentru refacerea depozitelor de magneziu, apoi 2-3 comprimate (1-1,5 g orotat de magneziu) pe zi. Comprimatele trebuie administrate cu puţină apă. Durata tratamentului trebuie să fie de cel puţin 6 săptămâni. Nu luaţi Magnerot dacă sunteţi alergic la orotatul de magneziu sau la oricare dintre celelalte componente ale Magnerot ori dacă aveţi afecţiuni ale rinichilor sau în prezenţa diatezei litiazice renale (calculi urinari alcătuiţi din fosfat de calciu, magneziu, amoniu). Acest medicament se poate elibera fără prescripţie medicală. Se recomandă citirea cu atenţie a prospectului sau a informaţiilor de pe ambalaj. Dacă apar manifestări neplăcute, adresaţi-vă medicului sau farmacistului.

WÖRWAG PHARMA

Sanatatea.Online
un proiect Sanatatea Press Group

Bun venit în lumea evenimentelor medicale exclusiv ONLINE!

Sanatatea.ONLINE vă oferă o experiență cu totul nouă a evenimentelor medicale desfășurate în spațiul virtual:

✔ CONGRESE

✔ CURSURI, WORKSHOP-uri și nu numai!

Platformă cu conținut dedicat exclusiv profesioniștilor din domeniul medical!

Direct din fața propriului dispozitiv: laptop, desktop, smartphone sau tabletă!

Accesați **https://sanatatea.online/learning/**
și creați-vă un cont de utilizator!

Descoperiți o nouă experiență în desfășurarea de evenimente ONLINE!

www.Sanatatea.ONLINE

Partener de încredere

SERVIER

40

2019

1973

ani de parteneriat

Servier Pharma S-Park
Str. Tipografilor nr. 11-15, Corp A, etaj 3,
Sector 1, Bucureşti
Tel.: 021-528.52.80; Fax: 021-528.52.81,
www.servier.ro

Neurossen® 300 mg

Benfotiamină

Eliberează-te din capcana neuropatiei diabetice!

WÖRWAG PHARMA

NSO/10.16/085